On the ribosomal proteins and the DNA Binding protein II from Thermus thermophilus, Thermus aquaticus, Bacillus caldolyticus and Clostridium pasteurianum

Inaugural-Dissertation
zur Erlangung des Doktorgrades
des Fachbereichs Chemie
der Freien Universität Berlin

vorgelegt von
Rainer Zierer
aus Moringen

(~~1984~~)
1985

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1. Introduction and Objective of the Thesis

The evolution of living cells has been studied by comparison of homologous macromolecules from various organisms. Early work in this field (1, 2) showed that the hemoglobins (1) from different mammals have similar primary structures but not identical. The function of these macromolecules was not altered throughout evolution, although changes in sequence took place (1, 2). This can be explained by the conservative substitution of amino acids, which means that the alteration of the primary structure was among one class of amino acids, such as neutral or charged residues. Through the sequence analysis of homologous molecules it has been learned that the differences in the primary structure of two proteins are related to the time which has elapsed since they orignated from a common ancestor. The comparison of homologous hemoglobins allowed the eludication of a phylogenetic tree. Since hemoglobin molecules are only found in higher organisms, such as mammals, it is not possible to study the evolution of prokaryotes by extension of these studies. For this purpose, "molecules" which are common throughout all living cells, have been studied. Among these molecules are those present in ribosomes since protein biosynthesis takes place in prokaryotes as well as in eukaryotes. The ribosome translates the genetic information of the mRNA into proteins.

The ribosome is a complex mixture of RNA and protein molecules; the RNA accounts for 60% of the total molecular weight of the ribosome (3). There are two types of ribosomes, 70S and 80S ("S" refers to the Svedberg coefficient, which describes the migration of macromolecules in a gravitational field). The 70S particles are present in prokaryotes, chloroplasts and mitochondria, whereas 80S particles are responsible for protein biosynthesis in eukaryotes.

From the comparison of homologous ribosomal RNA or proteins from different organisms, we can see the substitution of nucleotides or amino acids which took place during the evolution. The nucleotide sequence of homologous rRNA molecules ("r" denoting ribosomal), or the amino acid sequence of homologous r-proteins can give information on the phylogenetic relationship of different organisms (4, 5). For the study of evolutionary relationships among prokaryotes and eukaryotes it is necessary to examine a wide range of homologous r-molecules.

The work described in this thesis is concerned with comparative studies of ribosomal proteins from different bacteria and a DNA binding protein which can also associate with ribosomes (6, 22). Through the characterization of homologous proteins, it should be possible to deduce a relationship among prokaryotes belonging to the Gram-positive and Gram-negative families.

The structure of the components of the 70S ribosomes from the Gram-negative enterobacterium Escherichia coli has been intensively studied in the last ten years (for review see 8 - 13). The proteins of these ribosomes have been compared with their homologous counterparts from different genera of the enterobacteria, such as Salmonella (14 - 17). A strong similarity of the individual proteins could be seen. The same relationship was found for the primary structures of homologous r-proteins from Escherichia coli, Bacillus stearothermophilus (18 - 20) and Bacillus subtilus (24).

The sequence of DNA binding protein II of Escherichia coli (21, 73), which is found attached to 70S ribosomes (22), has also been compared with homologous proteins from other organisms such as Bacillus stearothermophilus (23), Rhizobium meliloti (25), Pseudomonas aeruginosa (26) and Thermoplasma acidophilum (27). It has been shown that some parts of the primary structure have not changed during evolution. These conserved parts of the sequence are presumably important for the structure and/or function of the proteins. From the degree of sequence homology we can learn more about the relationships amoung those organisms. For example, the DNA binding proteins II (NS) from Escherichia coli show a strong similarity to the corresponding Bsb-protein from Bacillus stearothermophilus (59% homology) but less to that from Thermoplasma acidophilum (27% homology) (23). The determination of the complete sequences of r-proteins and the DNA binding protein II is necessary to understand the evolution of closely related prokaryotes.

An overview of the ribosomal components of Escherichia coli and the methods which have been developed for the structural investigation of these components have been given by H.-G. Wittmann (11, 12). In order to be able to investigate the structure of ribosomal proteins, pure ribosomes must be obtained which do not contain cytoplasmic proteins. However, this must be achieved without the liberation of r-proteins. For this purpose, the ribosomes are separated by gel filtration from cytoplasmic mole-

cules in the presence of high concentrations of salt (28). The purified 70S particles can be separated into 30S and 50S subunits (29) by centrifugation in the presence of low Mg concentrations. To obtain a detailed picture of the number, molecular weight and charge of the r-proteins, they may be extracted from the subunits with acetic acid (96) and analysed with a two-dimensional-gel electrophoresis system (30, 31). The small subunit of Escherichia coli contains 21 S-proteins ("S" denoting small) and a 16S-RNA; this particle has a molecular weight of approximately 850.000 (10, 11). The large subunit contains 34 L-proteins ("L" denoting large), one 5S and one 23S RNA molecule. This subunit has a molecular weight of approximately 1.450.000 (10, 11). The complete amino acid sequences of 53 different proteins (L7 is equal with L12; S20 with L26) has been determined mainly by B. Wittmann-Liebold and her coworkers during the last decade (for review see H.-G. Wittmann, 8, 10, 11). The average molecular weight of proteins from the small subunit is about 16.600; the largest is 61.159 (S1) and the smallest 9.553 (S21), respectively. Proteins from the large subunit exhibit an average molecular weight of 13.5000, the largest one with 29.146 (L2) and the smallest one with 5.381 (L34). Most of the ribosomal proteins are rich in basic residues, such as lysine and arginine. Some N-termini are blocked by methylation or acetylation; these modifications occur after translation.

For the investigation of the secondary structure of r-proteins, new extraction and purification methods have been developed. They avoid the use of denaturing conditions, such as urea, low pH and lyophilization (32, 33). Groups of ribosomal proteins were extracted by a stepwise increase of salt concentration at neutral pH. By this means the intermolecular bonds between RNA and proteins are gently disrupted.

For the study of the secondary structure of proteins, circular dichroism measurements have been performed (34). Such studies have revealed that most of the 30S proteins are rich in α-helix and contain a small amount of β-sheet, whereas the proteins from the large subunit are more diverse, especially in their α-helix-content, and almost all of the 50S proteins are rich in β-sheet.

Studies on the internal structure of proteins, which is stabilised by the interaction of hydrophobic and aromatic residues, have been performed by proton magnetic resonance spectroscopy (PMR). The intramolecular inter-

actions between apolar methyl and methylene protons of valine, isoleucine or leucine and aromatic residues (phenylalanine, tyrosine, tryptophane or histidine) are revealed by the apperance of resonances shifted by ring-current effects to the high-field region of the PMR-spectrum (0 to -1 ppm) and by broadened, as well as perturbed, resonances at the low-field aromatic region (6 to 8 ppm). PMR studies on r-proteins from both the small and large subunits of Escherichia coli isolated under non-denaturing conditions show compact tertiary structures (35 - 37).

To obtain a more detailed knowledge of the structure of the purified r-proteins, crystallization and X-ray analyses have been performed. Using X-rays with a wavelength of about 0.1 nm to generate a diffraction pattern from a crystal, it is possible to deduce the arrangement of individual atoms in the molecules from which the crystal is formed. This technique is certainly time consuming, since several sets of crystals are required to obtain enough diffraction information. Some of the crystals have to be modified by binding heavy metal atoms to specific sites. Furthermore, growth of protein crystals is an empirical art, and so far r-proteins of the mesophile Escherichia coli are difficult to crystallize. The crystals of protein L29 diffract poorly and are therefore not suitable for X-ray analysis. The protein crystal L24 is stable and diffracts well (3 - 4 Å) when examined below °4C (private communication Dr. J. Dijk). A C-terminal fragment of protein L12 is the only r-protein of Escherichia coli for which the structure has been determined by X-ray analysis (39, 40).

In some instances it has been found that proteins of thermophilic organisms are more readily crystallized than their counterparts from mesophilic bacteria. The crystallization and tertiary structural determination by X-ray analysis of enzymes such as glyceraldehyde-3-P-dehydrogenase from Bacillus stearothermophilus (41) or superoxide dismutase from Thermus thermophilus (42) has given information concerning their structure and mechanism of action. Since these enzymes from thermophilic organisms can be crystallized more readily, it is reasonable to expect that the r-proteins from these organisms (which must also be stable under the same extreme conditions) might also be easier to crystallize.

The protein L9 was the first intact r-protein which was crystallized from Bacillus stearothermophilus (38). The X-ray analysis of these crystals is still under way at a resolution better than 3 Å. Protein crystals of the

pentameric $(L_{12})_4/L_{10}$-complex are now available (43). The proteins L6 (44) and S5 (45) from Bacillus stearothermophilus have been crystallized and studied at low resolution. Protein L30 yielded crystals suitable for a complete X-ray analysis and determination of the tertiary structure (46). As is typical of globular proteins, L30 is compact with a hydrophobic interior and a surface populated with charged residues. The overall topology consists of a 3-stranded, antiparallel beta-pleated sheet with 2 intervening α-helices. The same type of structure was seen in the carboxy terminal fragment of L12 (43), and is unique for these two proteins.

The work described in this thesis is concerned with the evolutionary relationship of Gram-negative and Gram-positive bacteria, namely with the thermophiles Thermus thermophilus, Thermus aquaticus, Bacillus caldolyticus and the mesophilic Clostridium pasteurianum. The ribosomal proteins from these organisms were compared with the corresponding molecules from Escherichia coli and Bacillus stearothermophilus. From sequence data on the 5S rRNA of Thermus thermophilus (74) and Thermus aquaticus (80) it is known that the two thermophilic organisms are closely related. Their 5S rRNAs contain 120 nucleotides, which is characteristic of the Gram-negative family (5), but the sequences reveal more homology to 5S rRNA from the Gram-positive Bacillus stearothermophilus (70%) than to that from Escherichia coli (64%). Thus, it would be interesting to determine how the ribosomal proteins of the two Gram-negative, thermophilic organisms are related to the proteins of Bacillus stearothermophilus and Escherichia coli.

Bacillus caldolyticus is related to Bacillus stearothermophilus (63, 97, 98), but the degree of relationship between those two organisms is not known, since the primary structure of the 5S rRNA from Bacillus caldolyticus has not yet been determined. Therefore, it was of interest to analyse the ribosomal proteins from the two organisms by two-dimensional-gel electrophoresis to study their relationship.

The 5S rRNA from Clostridium pasteurianum contains 116 nucleotides which is characterstic of the Gram-positive family. It has a homology of 72% to the corresponding molecule from Bacillus stearothermophilus (5). The similarity of the two organisms should be reflected by their r-proteins.

From the crystallographic data of the DNA binding protein II from Bacillus stearothermophilus (Bsb), it has been assumed that this protein binds to the sugar backbone of DNA (48, 75). It also has been found to be associated to ribosomes, but its function on the organelle is unknown. Homologous proteins have been isolated from other organisms (21, 25, 26, 27). From sequence information on this protein it is possible to deduce an evolutionary relatonship between the organisms concerned. Therefore, it was of interest to isolate the corresponding proteins from Thermus thermophilus, Thermus aquaticus, Bacillus caldolyticus and Clostridium pasteurianum for both evolutionary and functional studies since less is known about the structure of these molecules from extreme thermophiles.

Abbreviations

A_{260} units:		the amount of substance in 1 ml of solution giving an absorbance of 1.0 when measured at 260 nm with a pathlength of 1 cm.
APG	:	aminopropylglass
APS	:	amoniumperoxodisulfate
B.	:	Bacillus
BAM	:	Benzamidine hydrocloride
Bicine	:	N,N-bis(2-hydroxyethyl)-glycine
Bisacrylamide	:	N,N'-methylenebisacrylamide
C.	:	Clostridium
CM	:	carboxymethyl
DABITC	:	4-N,N-dimethylaminoazobenzene-4'-isothiocyanate
DABTH	:	4-N,N-dimethylaminoazabenzene-4'-thiazolinone
DABTZ	:	4-N,N-dimethylaminoazobenzene 4'-thiazo
DMF	:	dimethylformamide
DMS	:	Deutsche Microbiol. Sammlung, Göttingen, FRG.
DSS	:	sodium 2,2,dimethyl-2-silanpentanesulfonate
DTE	:	dithioerythriol
EDTA	:	ethylenedinitrilotetra acetic acid tetrasodium salt
Fluorescamine	:	4'-phenylspiro[furan-2(3H],1 phthalan]-3,3'-dione
HEPES	:	4(2 hydroxyethyl)-1-piperazinethanesulfonic acid
MPD	:	2-methylpentan-2,4-diol
Ninhydrine:		1,2,3 triketohydrindene-monohydrate
pBR 322	:	plasmid DNA, was a gift from Dr. Lanka, Abt. Schuster, Max Planck Institut für Molekulare Genetik, Berlin.
PITC	:	phenylisothiocyanate
PMSF	:	phenylmethanesulfonylfluoride
Putrescine:		1,4-bis-amino-butan
SDS	:	sodium dodecyl sulfate
Spermidine:		[N-(3-aminopropyl)-1,4-bis-amino-butan]x3HCl
TCA	:	trichloroacetic acid
TEMED	:	N,N,N',N'-tetramethylethylendiamine
TFA	:	trifluoroacetic acid
Th.	:	Thermus
TP	:	Total protein extract
Tris	:	trishydroxymethyl-amino-ethane

Chemicals

Acrylamide, DTE, Bisacrylamide, Coomassie Brilliant Blue R-250, Putrescine, Spermidine, Fluorescamine: Serva, Feinbiochemica, Heidelberg;

Hepes, Bicine, BAM, PMSF, deoxychloate: Calbiochem, San Diego, California, USA;

Millipore S.A., 67120 Molsheim, France, Filtertype HA, pore size: 0.45 µm)

TEMED, DABITC: Fluka AG. Switzerland;

DNase (RNase free), Trypsin-TPCK: Worthington Biochemical Corp., New Jersey, USA;

Polycytidylic acid, P-L Biochemicals, Inc., Milwaukee, Wis., USA;

CM-Sephadex-C-25, Sephadex-G-50-superfine, Sephacryl-S-300, Sephacryl-S-200, Heparin-Sepharose-Cl-6B: Pharmacia, Uppsala, Sweden;

All other chemicals were from Merck, Darmstadt, FRG, and were of the highest purity available (pro analysis).

2. Methods

All operations were carried out in the temperature range of 0°C - 4°C. Proteins, RNA and bacteria were stored at -70°C. To protect the proteins against oxidation and proteolytic degradation, all buffer solutions contained 2-mercaptoethanol, benzamidine and phenylmethanesulfonylfluoride at concentrations of 6 mM, 0.1 mM and 0.05 mM, respectively.

2.1. Growth of Thermus thermophilus cells

The thermophilus cells were obtained from DMS. A preculture of cells was inoculated overnight in a 4 l flask. The flask was kept in an oven at 80°C. The solution was continuously aerated using 300 ml oxygen/min. The nutrient and buffer compositions were used as described in (95).

1 g nitrilotriacetic acid
1 ml $FeCl_3$ solution (0.3 g/l)
0.6 g $CaSO_4 \cdot 2H_2O$
1.0 g $MgSO_4 \cdot 7H_2O$
0.8 g NaCl
1.0 g KNO_3
3.0 g $NaNO_3$
1.0 g NaH_2PO_4
10 ml Solution A
in 1000 ml H_2O.

0.1% (W/W) glucose
0.3% (W/W) peptone

Solution A was adjusted to pH 7.2 with 1 N NaOH,
and contained per litre:
0.5 ml H_2SO_4 conc
2.28 g $MnSO_4 \cdot 2H_2O$
0.5 g $ZnSO_4 \cdot 7H_2O$
0.5 g H_3BO_3
0.025 g $Cu\ SO_4 \cdot 5H_2O$
0.025 g $Na_2MoO_4 \cdot 2H_2O$
0.045 g $Co\ Cl_2 \cdot 6H_2O$

The preculture was grown up to an optical density of 0.6 at 650 nm. The solution was transferred to a 100 l fermenter, equipped with a stirring system and continuous air supply. The buffer and nutrient conditions were

the same as in the preculture. The airstream was increased to 6 l/min. The bacteria started to grow after a log phase of 4 - 5 hr. The pH and optical density of the solution were measured every 30 min. During the log phase, the pH was adjusted by addition of 1 M NaOH-solution since the bacteria produced an acid compound. There was no improvement of growth rate by further addition of glucose. At the mid-log phase, the bacteria were treated with ice-cold water to stop further growth. The bacteria were harvested using a continuous flow centrifuge and stored at -70°C until required.

2.2. Preparation of 70S ribosomes

The purification of ribosomes was carried out according to the procedure of Jelenc (28) with several modification (48). The buffer solutions used were:

buffer 1: 10 mM Hepes-KOH, pH 7.5
100 mM KCl
10 mM $MgCl_2$

buffer 2: 5 mM $CaCl_2 \cdot 2H_2O$
50 mM $MgCl_2 \cdot 6H_2O$
50 mM NH_4Cl
950 mM KCl
10 mM Spermidine
80 mM Putrescine

Unless stated otherwise this stock solution was diluted 10-fold with H_2O.

buffer 3: 1 l of buffer 2, stock solution
1 l of 200 mM Hepes pH 7.5 (KOH)
8 l H_2O dest.

To avoid the oxidation of sulfhydryl groups of proteins, dithioerythriol (DTE) was added to a final concentration of 1 mM, prior to use.

To remove cytoplasmatic as well as DNA binding proteins from the 70S ribosomes, ammoniumchloride was added to the final concentration of 1 M.

buffer 4: 20 mM sodium phosphate, pH 6
100 mM NaCl

buffer 5: 20 mM sodium phosphate, pH 6
350 mM NaCl

500 g of bacteria were thawed overnight and washed twice with 500 ml buffer 1. After centrifugation at 7000 xg, the pellet was suspended in 500 ml buffer 2. 2 mg of DNase (RNase-free, Worthington) were added in order to reduce the viscosity of the solution while breaking the cells. The cells were mixed in a Waring blender to obtain a homogenous suspension and then broken by passage through a French press at a pressure of 10.000 psi. This was performed twice, with intensive cooling in ice between the two cycles. To remove unbroken cells and cell debris, the solution was centrifuged in a Sorvall rotor SA 600 at 30.000 xg for 30 min. The supernatant was removed and treated with solid $(NH_4)_2SO_4$ to a concentration of 210 g/l and stirred. The pH of the solution was adjusted to 7.5 with NaOH. The stirring was continued for 30 min. and precipitated protein removed by centrifugation. To separate membrane lipids, the solution was passed through a Sephadex-G-25-column. The column dimensions were 30 cm x 10 cm and the flow rate 1 l/hr. For further purification, the clear, yellow breakthrough from the G-25-column was loaded onto a Sephacryl-S-300-column with dimensions of 140 x 9 cm. A flow rate of 0.45 l/hr was used. Fractions of 0.25 l were collected and 10 µl from each fraction analysed by SDS-slab-gel electrophoresis (55). Ribosomes elute after 3.25 l, whereas the DNA binding protein II elutes after 5 l. The fractions containing ribosomes were pooled, the total volume measured and polyethyleneglycol 6000 added with stirring to a final concentration of 11% W/V (49). The ribosomes were precipitated within 30 min. and were collected by centrifugation in a GSA rotor at 7000 rpm for 20 min. The clear supernatant was discarded. The pellet was suspended in 100 ml of buffer 2 and left to dissolve without stirring, then frozen until required.

2.3. Preparation of subunits from 70S ribosomes

Subunits were prepared from 70S ribosomes by zonal centrifugation. The following buffer solutions were used:

solution 1: 800 ml 1 M $K_2HPO_4 \cdot 3H_2O$, pH 8.8
200 ml 1 M KH_2PO_4, pH 4.05
the pH of this solution is 7.5

solution 2: 0.1 l solution 1
10 ml 1 M $MgCl_2 \cdot 6H_2O$
5 ml 2-mercaptoethanol made up to 10 l in water

solution 3: (38% W/W Sucrose, Ultrapure, Bethesda Res. Lab.)
2280 g Sucrose
3650 ml H_2O
120 ml solution 1
12 ml 1 M $MgCl_2$
6 ml 2-mercaptoethanol

solution 4: (48% W/W Sucrose, Ultrapure, Bethesda Res. Lab)
2880 g sucrose
3120 ml H_2O

70S ribosomes at a concentration of 200 A_{260} units/ml were dialysed overnight against solution 2 in order to dissociate the subunits. Samples of 40 ml (8000 A_{260} units) were applied with a reverse 6% - 0% sucrose gradient onto a prepared 6% - 38% sucrose gradient in a Ti 15 zonal rotor which was obtained by mixing solutions 2 and 3. The subunits were sedimented for 15 hr. at 50.000 g. After slowing the centrifuge down to 3000 rpm, 15 ml fractions were collected by displacing the gradient from the rotor with 48% sucrose solution. The separation of subunits was monitored by their absorption at 280 nm and precipitated as described for 70S ribosomes. The 30S and 50S subunits were finally resuspended in buffer 2 at a concentration of 200 A_{260} units/ml. They were kept frozen until required.

2.4. Extraction and purification of DNA binding proteins

The binding proteins were located in post-ribosomal fractions from the Sephacryl-S-300-column by their migration on the SDS-slab-gel electrophoresis. The proteins appear as a sharp band at the low molecular weight part of the gel.

Fractions containing the proteins were pooled and dialysed thoroughly against buffer 4. The yellow solution, freed of precipitated proteins, was applied to an ionic exchange column and, for further prufication, on a gel filtration column (Sections 2.4.1. - 2.4.4.).

2.4.1. CM-Sephadex-C-25 ionic exchange chromotography

The solution containing the binding protein was loaded on a CM-Sephadex-C-25-column with dimensions of 4 cm x 10 cm. The breakthrough fraction was checked for unbound protein by SDS-slab-gel electrophoresis. A linear gradient of NaCl, 0.1 M - 1 M, in 0.02 M Na_2HPO_4, pH 7 (NaOH), with a total volume of 1 l was used for elution. The flow rate was 4 ml/hr. Fractions of 4 ml were collected and 5 µl of every second fraction was checked for protein content by SDS-slab-gel electrophoresis (Section 2.11.1.).

2.4.2. Heparin-Sepharose-Cl-6B-affinity-chromatography

Fractions containing the DNA binding protein, after dialysis (see above), were applied to a Heparin-Sepharose-Cl-6B-affinity-column, which was packed in a disposable syringe. The dimensions of the gel bed were 1 cm x 2 cm. The ionic exchanger was equilibrated with 20 ml of buffer 4 and the protein eluted with a linear gradient of NaCl (0.1 M - 1.0 M in the presence of 0.02 M Na_2HPO_4, pH 7). The gradient volume was 150 ml and the flow rate 4 ml/hr. An aliquot of 20 µl from each second fraction was analysed by SDS-slab-gel electrophoresis.

2.4.3. Sephadex-G-50-gel filtration

In order to avoid the aggregation of proteins, the protein mixture was dialysed against buffer 5 and then applied to a Sephadex-G-50-superfine-column for further purification. The column dimensions were 150 x 5 cm. The flow rate was 30 ml/hr. Fractions of 15 ml were collected and 20 µl of

each second fraction analysed for protein content by SDS-slab-gel electrophoresis. The absorbance of the eluate at 230 nm was read continuously.

2.4.4. Sephacryl-S-200-gel filtration

For amino acid analysis and primary structure studies, proteins which were still not pure after the preceding steps were separated further on a Sephacryl-S-200-column in the presence of 20% acetic acid. The column dimensions were 150 x 5 cm and the flow rate was 30 ml/hr. Fractions of 10 ml were collected and aliquots of 20 µl from each fraction analysed for protein as mentioned above.

2.5. Concentration, determination of protein concentration and storage of proteins

To preserve the native structure of proteins they were concentrated avoiding denaturing steps such as lyophilization, trichloroacetic acid or salt precipitation. The pure protein fractions were dialysed against buffer 4 and loaded into a small syringe, packed with 1 ml of CM-Sephadex-C-25. After checking the breakthrough fractions for unbound proteins, the protein was eluted three times with 1 ml 1.5 M NaCl. The three fractions were dialysed against buffer 5. By this procedure, protein solution could be reduced to volumes of 2 - 3 ml, with a protein concentration range of 4 - 30 mg/ml.

Determination of protein concentrations was carried out according to Udenfriend et al. (50): 240 µl of 50 mM tetraborate-buffer was mixed with 40 µl of protein solution in an Eppendorf tube. 50 µl of fluorescamine solution in aceton, 6 mg/ml was added and mixed rapidly. After 10 min. an aliquot of 200 µl was transferred to a 250 µl optical cell (3 mm path length) and the fluoresence measured in an Amino-Bowman spectrofluorometer (exitation at 390 mm, emission at 475 nm). A lysozyme standard was used to obtain a calibration curve in the range of 5 - 50 µg. After the concentration step, the proteins were dialysed against buffer 5 and stored in portions of 0.5 ml at -70°C until required.

2.6. Acetic acid extraction of ribosomal proteins (96)

2000 A_{260} units of either ribosomes or subunits were processed. The solutions were dialysed against buffer 2 in the presence of 0.1 M $MgCl_2$. Two

volumes of cold acetic acid were added slowly. After stirring for 2 hr. on ice, the rRNA and proteins were separated by centrifugation for 1 hr. at 30.000 g. The rRNA pellet was washed with 66% W/V acetic acid and pelleted again by centrifugation. The supernatants were combined, dialysed against 1% acetic acid, lyophilized and stored frozen.

2.7. Acetic acid/urea extraction of total ribosomal protein, 5S and 16S rRNA

The extraction was carried out using the Hochkeppel procedure (51) with a few modifications; since I was interested in the 5S as well as 16S rRNA for protein binding experiments, a larger amount of ribosomes was employed. The following buffer solutions were used:

buffer 6: 30 mM Tris-HCl, pH 8
20 mM $MgCl_2$

buffer 7: 20 mM Hepes-KOH, pH 7.4
0.5 mM $MgCl_2$

buffer 8: 500 mM NaCl
10 mM $MgCl_2$
10 mM Tris-HCl, pH 8

25.000 A_{260} units of 70S ribosomes were dialysed extensively against buffer 6 and diluted with the same buffer to a concentration of 100 A_{260} units/ml. An equal volume of 8 M urea was added and the solution stirred for 15 min., followed by addition of solid Mg $(OOCCH_3)_2$ to a final concentration of 0.8 M. After stirring for 15 min., three volumes of cold acetic acid were added slowly and the suspension was stirred for 1 hr. The precipitated rRNA was pelleted by centrifugation at 30.000 g for 20 min.; the supernatant, containing TP-70, was dialysed against 1% acetic acid and lyophilized. The rRNA pellet was suspended in half the initial volume of buffer 7. After addition of solid Mg $(OOCCH_3)_2$ to a final concentration of 1 M, the solution was stirred for 15 min. Four volumes of cold acetic acid were added slowly and stirred for 2 hr. The rRNA was pelleted for 20 min. at 30.000 g. The supernatant was discarded and the rRNA pellet dissolved in 100 ml buffer 7 with shaking overnight. As the rRNA was not completely dissolved, the solution was centrifuged and the pellet, contain-

ing the 23S rRNA, was discarded. The supernatant containing the 5S and 16S rRNA was treated with 2.5 Vol. of ice-cold ethanol and kept at -20°C, then centrifuged for 30 min. at 8000 xg. The pellet was dissolved in 20 ml of buffer 8. All the rRNA went into solution.

2.8. Purification of 5S and 16S rRNA

Separation of the 16S and 5S rRNA was performed on a Sephacryl-S-200-column. The column (150 x 5 cm) was equilibrated with buffer 8. The flow rate was 20 ml/hr. 5 ml fractions were collected and an aliquot of 30 µl from each second fraction was analysed by SDS-slab-gel electrophoresis as well as by absorption measurement at 260 nm. Fractions containing pure rRNA were pooled, precipitated with 2.5 Vol. of ice-cold ethanol and stored overnight at -20°C. After centrifugation at 8000 xg for 20 min., the pellet was dissolved in buffer 2 which had been previously sterilized. The concentration was determined by measurement of the absorbance at 260 nm (1 A_{260} unit is equivalent to 40 µg RNA).

2.9. Assay for protein/nucleic acid complex formation

The isolated proteins were checked for complex formation with DNA using a filter binding assay. Furthermore, the binding was confirmed by gel filtration of protein/nucleic acid complexes as well as of protein, ribosome and protein-subunit mixtures.

All buffer solutions were sterilized prior to use.

2.9.1. DNA filter binding assay

The filter binding assay was performed according to (52) with the following solutions:

buffer 9: 150 mM Na_3 Citrate
15 mM NaCl
pH 7.0 with citric acid

buffer 10: 5 M NaCl

buffer 11: ([^{3}H]-DNA)
340 µg/ml of cold, double-stranded plasmid DNA, pBR 322, which contains 4363 base pairs, was mixed with 7 µg/ml of [^{3}H]-DNA ($9 \cdot 10^6$ cpm)

For the binding assay, solution 11 was diluted 1:10 with H_2O prior to the experiment. 60 µl of the diluted solution 11 was mixed with 600 µl of buffer 9 and 316 µl of solution 10. The mixture was diluted with 4.424 ml H_2O. Increasing amounts of the isolated proteins (1, 2, 4, 10, 30 µg/50 µl buffer 9, respectively) were added and incubated 20 min. at 37°C. The protein bound DNA was separated from free DNA by absorption onto a Millipore filter under suction. The filter had been equillibrated with buffer 9 in the presence of 50 µg/ml bovine serum albumin. The filters were dried, treated with scintillation fluid and counted.

2.9.2. Sephadex-G-75-column assay

Protein nucleic acid complexes were prepared by incubating the nucleic acid with individual proteins for 5 min. at 40°C followed by incubation for 55 min. at 37°C in a volume of 100 µl - 200 µl of 350 mM NaCl, 10 mM $MgCl_2$ and 10 mM Hepes (NaOH, pH 7.4). Protein nucleic acid molar ratios of 8:1, 4:1, 2:1 and 1:1 were used. Ribosomes and subunits of ribosomes were incubated with increasing amounts of protein. The following molar protein/subunit and protein/ribosome ratios were used: 120:1, 80:1, 40:1. Prior to gel filtration, the mixtures were cooled to 4°C. 20 µl glycerol were added to the incubation mixture and the sample applied to a Sephadex-G-75-column (30 x 1.5 cm) at 4°C. The flow rate was 63 and 32 ml/hr. and was kept constant by a Pharmacia-P-3 peristaltic pump.

The void volume was 12 ml. Absorption of nucleic acids at 260 nm was measured continuously using a UV-monitor connected to a recorder. Fractions of 0.3 ml were collected and tested for nucleic acid-bound protein by precipitation with 5% trichloroacetic acid in the presence of 5 µl 0.5% sodiumdeoxycholate, followed by SDS-slab-gel electrophoresis.

2.9.3. Sephadex-G-50-column assay

Protein nucleic acid complexes were prepared as above except that buffer 2 was used since the ionic combinations of buffer 2 have been useful in in vitro translation systems (7). The mixture was packed on a Sephadex-G-50-column (40 x 1 cm) with a flow rate of 88 ml/hr. The void volume was 13 ml. Fractions were collected and analysed for protein as described.

2.10. Electron microscopy of protein/DNA complexes

To see the effect of the isolated proteins on the DNA structure, the nucleo-protein complexes were employed for electron microscopy studies.

Plasmid DNA RSF-1010 was a gift from Dr. Lanka. Protein and DNA in a weight ratio of 10:1 were incubated in 50 mM KCl, 0.5 mM DTE, 0.5 mM EDTA, pH 7.5 for 15 min. at 37°C. Glutaraldehyde was added to a final concentration of 0.1%. The mixture was incubated for a further 15 min. at 35°C and unbound protein was removed by Sepharose-4B-column chromatography in the presence of the incubation buffer (see above). The column dimensions were 6 cm x 0.4 cm. The electron microscopy was performed as described in (53, 54).

2.11. Gel electrophoresis of ribosomal proteins and rRNA

2.11.1. SDS-slab-gel electrophoresis (55)

SDS-gel electrophoresis was used for the detection of proteins in column eluates and for the determination of the purity of proteins. The following stock solutions were used to prepare the gel.

(A): 60 g acrylamide, 1.6 bisacrylamide in 200 ml H_2O
(B): 1.5 M Tris-HCl, pH 8.8
(C): 10% W/V SDS
(D): 0.5 M Tris-HCl, pH 6.8
(E): 0.05 M Tris, 0.38 M glycine, pH 8.8, 0.1% SDS

The separation gel contained: 50 ml of (A), 25 ml of (B), 1 ml of (C) and H_2O up to 99 ml. The spacer gel contained: 2 ml (A), 0.1 ml (C), 2.5 ml (D) and H_2O up to 5.3 ml. 25 ml of separation gel was mixed with 25 µl

TEMED and 250 µl of a 10% W/V amoniumperoxodisulfate (APS) solution and poured into the lower buffer reservoir to act as a seal. After polymerization, 50 ml of separation gel was mixed with 50 µl TEMED and 500 µl APS and slowly poured up to 4 cm from the top of the plates and overlaid with 0.1% SDS. After polymerization, 10 ml of spacer gel was mixed with 10 µl TEMED and 100 µl of APS solution and poured on top of the separation gel. A comb for 25 or 50 samples was inserted immediately. Samples containing 2 µg - 5 µg protein in 10 µl - 20 µl volume were mixed with 10 µl sample buffer (2.5 ml (D), 2 ml (C), 2 ml glycerol, 0.1% bromophenolblue solution and 3.2 ml H_2O). More diluted samples were first precipitated by addition of 5 µl 0.5% sodiumdesoxycholate and trichloroacetic acid to 5%. After standing on ice for 30 min. the samples were centrifuged for 1 min. at 12.000 rpm in a Beckman Microfuge. The pellet was washed with diethylether to remove residual TCA, then dissolved in 25 µl sample buffer. The gels were run in buffer (E) for 6 hr. at 90 V. The staining solution for proteins contains:

1 g Coomassie Brilliant Blue R250, 100 ml acetic acid, 500 ml methanol and 400 ml H_2O

RNA was stained in:

0.5% W/W methylene blue
0.25 M sodium acetate
0.25 M acetic acid
in 50% V/V ethanol

After staining for 1 hr. the protein gels were destained in a solution containing 100 ml acetic acid, 100 ml methanol and 800 ml H_2O. The RNA gels were destained in 30% V/V ethanol.

2.11.2. Mini-two-dimensional-gel electrophoresis

The identification and determination of purity of proteins was performed on two-dimensional-polyacrylamide-gel electrophoresis (30, 31). In the first dimension the proteins are separated according to their charge, whereas migration in the second dimension is largely dependent on their molecular weight. The following solutions were used for the first dimension:

Solution A: 4 g acrylamide
0.1 g N,N'-methylene-bisacrylamide
1.19 g Bis-Tris[bis[2-hydroxyethyl]imino-tris[hydroxymethyl]methane
36 g urea
0.19 g EDTA (ethylenedinitrilo-tetra-acetic acid)

in a total volume of 100 ml destilled H_2O. The pH of the mixture was adjusted to 5 with acetic acid. The gel mixture was filtered, degassed and stored at 4°C. The gel was polymerized as follows: to 10 ml of ice-cold gel mixture, 35 µl of TEMED and 100 µl of 10% W/V APS were added. After stirring, this was immediately poured into gel tubes (0.6 x 10 cm) and the gels were overlayered with 4 M urea solution.

electrode buffers: the upper and the lower buffers were prepared as 10-fold concentrated solutions

upper buffer: concentrations per litre:
20.9 g Bis Tris
45 ml glacial acetic acid (pH 4)

lower buffer: concentration per litre:
175 g potassium acetate
49 ml glacial acetic acid (pH 5)

Sample preparation: protein samples were dissolved in the sample loading buffer at 100 - 250 µg/100 ml (the sample loading buffer was prepared by dissolving in the upper electrode buffer urea and DTE in concentrations at 6 M and 10 mM, respectively). 1 - 2 drops of basic fuchsin (0.5 mg/ml) were added per 10 ml as tracking dye.

Electrophoresis in the first dimension: gels were placed in the electrophoresis chamber and both upper and lower electrode buffers added. Samples were then loaded onto the top of the gels. Electrophoresis was carried out at 4°C and at 1 mA per gel tube for the first 30 min. and then at 4 mA per gel tube until the dye reached the bottom (6 hr.).

Electrophoresis in the second dimension: after the run, the gels were taken out of the glass tubes and placed on the second-dimension-gel slabs (10 x 10 x 0.3 cm), prepared according to the procedure of Kaltschmidt and Wittmann (30) modified by Geyl et al., (31). The gel-mixture contained per litre:

372 g urea
186 g acrylamide
4.8 g N'-N'-methylenebisacrylamide
45 ml glacial acetic acid
10 ml 5 M KOH

The pH of the mixture was 4.5. The solution was filtered, degassed and stored at 4°C.

Electrophoresis in the second-dimension was carried out at 100 V for 12 hr. The gels were stained with Coomassie Brilliant Blue (R260) as mentioned.

2.12. Sequence analysis of proteins

2.12.1. Amino acid analysis

Samples containing approximately 2 - 5 nmoles protein were dissolved in 200 µl 5.7 M HCl (destilled twice), containing 0.02% 2-mercaptoethanol and were hydrolysed in evacuated sealed tubes for 20 hr. at 110°C. Under these conditions, not all amino acids are recovered quantitavely. Alkaline hydrolysis for separate estimation of tryptophane and determination of cysteine content were not performed. After drying, the samples were dissolved in 100 µl of sample buffer pH 2.2 (a standard Durrum sample buffer). After clarification of the solution by centrifugation at 12.000 rpm for 4 min. in a Beckman Microfuge, 20 µl were analysed in a Durrum D-500 amino acid analyser.

2.12.2. Manual Edman degradation

The N-terminal sequence of proteins, as well as the sequence of peptides, was performed according to the Edman degradation procedure as modified by Chang et al. (56). 5 - 10 nmoles of salt-free protein or peptide in a

glass tube were dissolved in 80 µl of 50% aqueous pyridine solution. The solution was then treated with 40 µl of DABITC in pyridine (2.82 mg/ml), the tube was flushed with N_2 and placed in a heating block at 52°C for 50 min. After the first coupling, 10 µl of PITC were added and the second coupling reaction was allowed to proceed at 52°C for 30 min. After the reaction, the excess reagents were removed by mixing the sample with three portions of 40 µl of heptane/ethyl acetate (1:1), followed by centrifugation. The organic phase was removed and discarded and the mixture then dried under a high vacuum. The dried residue was dissolved in 50 µl trifluoroacetic acid (TFA), flushed with N_2 and placed in a heating block at 52°C for 15 min. The sample was dried in a vacuum dessicator and dissolved in 50 µl of water. Extraction of the cleaved DABTZ-amino acids was performed by mixing with 200 µl butyl acetate followed by centrifugation. After removal of the organic layer, the peptide in the water phase was dried and subjected to the next degradation cycle. The butyl acetate was removed, the residue was dissolved in 50 µl 40% TFA and placed in the heating block at 52°C for 50 min. The sample was dried and redissolved in 5 µl ethanol.

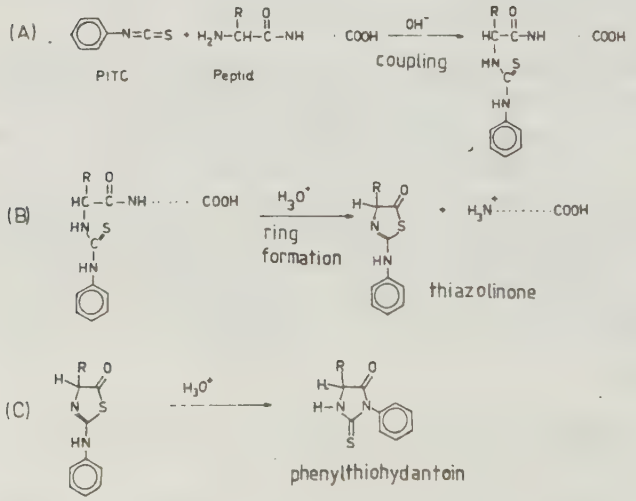


Fig. 1: Coupling cycles of amino acids in the presence of PITC, (A) Coupling of the NH_2-group with the dye, (B) cleavage of the peptide bond and ring formation of a thiazolinone, (C) reconversion of a thiohydantoin-derivative in the presence of TFA.

The identification of DABTH-amino acids was carried out on polyamide sheets (2.5 x 2.5 cm). For the first dimension acetic acid/water (1:2) was used, and for the second: toluene:n-hexane:acetic acid in the ratio 2:1:1. 1 - 3 µl sample was co-chromatographed with blue synthetic marker and after separation the plates were dried and exposed to HCl vapour in order to visualize the amino acid spots. They were then identified by relating their positions to a synthetic marker dye.

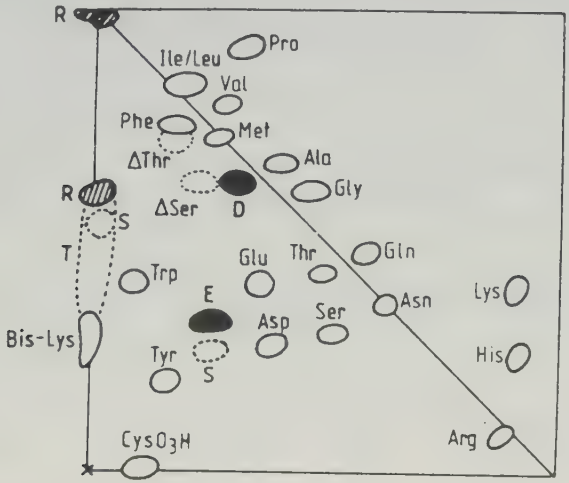


Figure 2. *Two-dimensional separation of DABTH-amino-acids.* A standard mixture of DABTH-amino-acids (50–100 picomole of each) are applied onto polyamide sheets (2.5 × 2.5 cm, from Schleicher and Schüll, Dassel) and developed in the following solvent mixtures: solvent I: 33% acetic acid; solvent II: Toluene/n-hexane/acetic acid (2:1:1, by vol). D and E are synthetic blue markers obtained by the reaction of diethylamine and ethanolamine, with DABITC [illegible]; R means remainder of reagent; T, S, ΔThr, and ΔSer are degradation products of the derivatives of threonine and serine, respectively. DABTH-Leu and -Ile are separated according to Yang (64) on silica gel sheets (HPTLC Kieselgel 60 F_{254}, 10 × 10 cm, from Merck, Darmstadt) in chloroform/methanol (50:2, by vol).

2.12.3. Solid-phase sequencing

This method was performed according to the procedure of Horn and Laursen (57), with the modifications of Wittman Liebold and Kimura (58). The technique of solid-phase Edman degradation relies on efficient procedures for attaching peptides or proteins to amino-substituted insoluble supports, such as aminopropylglass. Cleavage of proteins at methionine with BrCN

yields peptides with a homoserine at the C-terminus which can be converted to a homoserine lactone by treatment with TFA. The lactone is, when sufficiently activated, able to couple to aminopropylglass. The carboxyl groups of other peptides have to be activated prior to attachment to aminopropylglass (see below).

2.12.3.1. Attachment of peptides via C-terminal carboxyl-groups (58)

5 - 10 nmoles of the peptides were transferred to a solid-phase tube and dried in vacuo over P_2O_5, 100 µl of anhydrous TFA were added, the solution flushed with N_2 and incubated for 15 min. at room temperature. The sample was dried in vacuo (30 min.) and 2 ml of 1-ethyl-3 (3-dimethylaminopropyl)-carbodiimide/HCl, dissolved in 200 µl dimethylformamide (DMF) was added. 10 mg aminopropylglass beads, prewashed in methanol and DMF were then added. The mixture was flushed with nitrogen, degassed and kept at room temperature for 30 - 60 min. with gentle stirring. After centrifugation the beads were washed with water and methanol (2 x 2 ml). To saturate the remaining sites on the beads 20 µl PITC in 100 µl DMF and 50 µl of pyridine in 200 µl DMF were introduced to the reaction vessel. The mixture was flushed with N_2, degassed and incubated at 50°C for 20 min. The beads were centrifuged, washed with DMF and methanol (2 x 2 ml) and dried under vacuum.

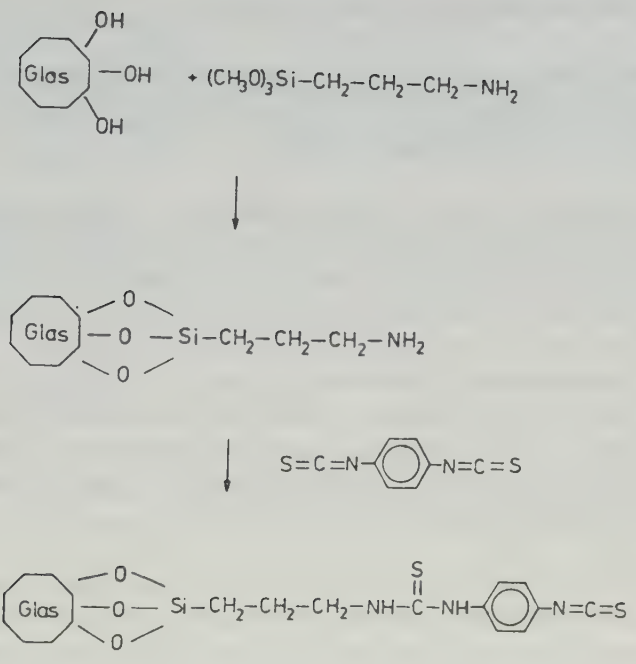


Fig. 3: The glass support is reacted with γ-aminopropyl-trimethylsilan (APG) and the amino-groups of the insoluble glass support are activated with PITC.

2.12.3.2. Attachment of peptides via a homoserine lactone residue (58)

10 nmoles of homoserine-containing peptide were transferred to a solid-phase tube and dried in vacuo over P_2O_5. 300 µl of anhydrous TFA were added, the tube flushed with nitrogen, and incubated at room temperature for 1 hr. By this procedure all the homoserine residues are converted to the lactone. After this, the sample was dried in vacuo.

The peptide with the lactone at the C-terminal was dissolved in 300 µl DMF, 10 mg of APG (prewashed in DMF) and 50 µl of triethylamine were added and the mixture was flushed with N_2 degassed in vacuo and incubated at 45°C for 2 hr. The sample was centrifuged and dried in vacuo.

2.12.3.4. Manual solid-phase Edman degradation

The manual solid-phase sequencing method is an improved version of the Chang et al. procedure (56). The reaction was carried out in 1 x 7 cm Edman tubes with gentle magnetic stirring.

400 µl 50% pyridine and 200 µl DABITC-solution (2.3 mg/ml pyridine) were added to the peptide attached to the glass. 20 µl of PITC were transferred to the tube, the mixture flushed with N_2 and incubated at 55°C for a further 30 min. (in the first cycle the DABITC-solution was omitted and replaced by 50 µl of PITC to complete the coupling reaction). The solution was centrifuged and the supernatant removed. The beads were washed twice with 500 µl of methanol. After this the beads were dried in vacuo and 200 µl of anhydrous TFA were added to the vessel. The mixture was flushed with nitrogen, incubated at 55°C for 8 min. to complete the cleavage and dried in vacuo.

The dye coupled amino acid was removed by two extraction steps with methanol (400 µl and 200 µl methanol, respectively). The combined methanol phases were dried under vacuum for 35 min. and the conversion of the thiazoline to the thiohydantoine derivative was carried out by addition of 40 µl TFA and 60 µl H_2O. The mixture was flushed with nitrogen and incubated at 50°C for 50 min. After drying under vacuum, the identification was carried out as described in Section 2.12.2.

2.12.4. Cleavage of proteins

2.12.4.1. Tryptic digestion

Proteins and peptides were digested with trypsin which cleaves at the carboxyl-group of arginine and lysine. The optimum pH of the reaction at 37°C is 7.9. The enzyme had been treated with 1-chloro-4-phenyl-3-tosylamido-2-butanol to avoid side reactions due to chymotryptic activity.

For preparative digestion, 2 - 4 mg of protein were dissolved in 2 ml N-methyl-morpholine pH 7.9 and preincubated at 37°C for 10 min. The enzyme (3.5 units/mg) was dissolved in H_2O and added to the protein in a ratio of 1:50 by weight. After 4 hr. the reaction was stopped by freezing and lyophylization.

2.12.4.2. Peptic digestion

Cleavage of proteins with pepsin occurs at the carboxyl-groups of phenylalanine, tyrosine, glutamic acid, alanine and leucine and also at the aminogroup of tyrosine.

2 - 4 mg of lyophilized protein was dissolved in 1 ml of 10% aqueous formic acid. Pepsin, dissolved in the same solvent, was added at a protein/enzyme ratio of 1:100 (W/W). After 4 hr. at 37°C the reaction was stopped by diluting 1:10 with H_2O and lyophilization.

2.12.4.3. Cyanogen bromide cleavage

The cleavage was performed according to Gross (59). Cyanogen bromide is capable of cleaving thioesters. The action of BrCN upon proteins is unique in its selective attack on methionine. The peptide bond is cleaved and the C-terminal methionine converted to homoserine or homoserine-lactone.

About 5 mg of protein was dissolved in 450 µl of 70% formic acid. BrCN was dissolved in 70% formic acid to a concentration of 200 mg/ml. 50 µl of this solution was added to the protein (BrCN was in a 20-fold molar excess with respect to methionine). The reaction vessel was wrapped in aluminium foil and stirred overnight at room temperature. The reaction was stopped by diluting 10-fold with H_2O and lyophilization.

2.12.5. Separation and identification of peptides

The peptides generated by the enzymic digestions were separated on 2-D thin layer plates. Separation in the first dimension is on the basis of charge using high voltage electrophoresis and in the second dimension on the basis of hydrophobicity using chromatography:

buffer A: pyridine:acetic acid:H_2O in the ratio of 25:1:225 (pH 6.5)
buffer B: n-butanol:pyridine:glacial acetic acid:H_2O in the ratio of 90:60:18:72.

The silica gel sheet (20 x 20 cm) was uniformly wetted with buffer A. Excess moisture was removed with clean filter paper. The enzymic digest

(2 - 4 mg) was dissolved in 50 µl H_2O. For peptide mapping 12 µl of the sample was applied to each sheet. The electrophoresis was carried out at 360 Volt/20 cm. The temperature was kept at 20°C by a water cooling system. After 2 hr. the sheets were dried at room temperature and the chromatography performed. Development was carried out for 5 - 6 hr. and the silica sheet dried with a hair dryer.

The 2-D plate was stained with fluorescamine, which reacts with the N-terminal and ε-amino-group of peptides. The peptides can be viewed under a UV-lamp as blue fluorescent spots.

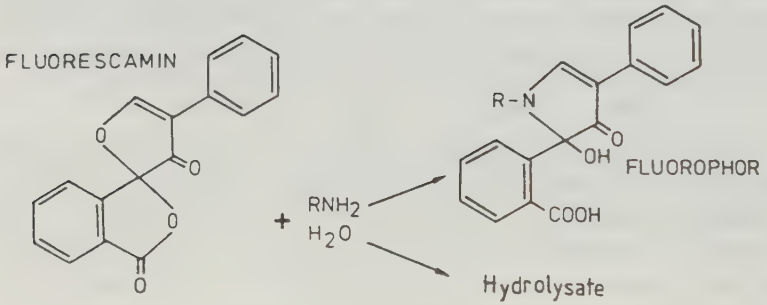


Fig. 4: For staining, the silica sheet was first wetted with pyridine (5% in acetone) and secondly with fluorescamine solution (10 mg fluorescamine per 500 ml acetone).

An alternative staining procedure was to use ninhydrin (triketohydrindenhydrate). The primary amino-groups of proteins, peptides and ε-amino-groups of amino acids couple to the dye to form a complex which is visible in normal light.

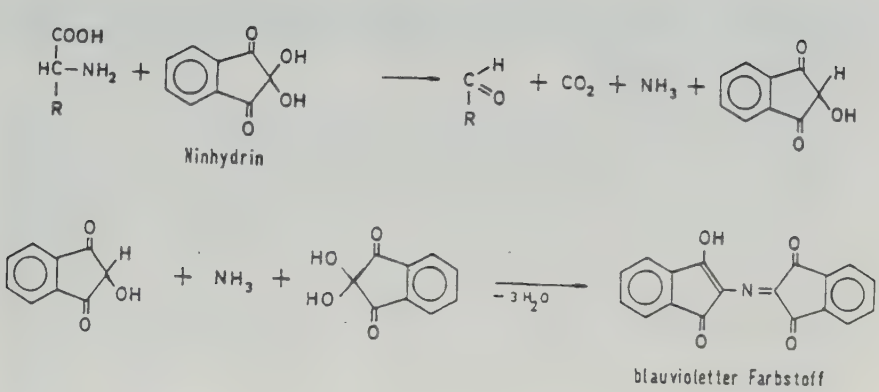


Fig. 5: Reaction scheme of ninhydrin coupling to primary amino-groups.

The contents of the staining solution were as follows:

500 mg ninhydrin
5 ml 4-ethyl-2-methyl-pyridine
20 ml acetic acid

diluted with ethanol to 500 ml.

For sequencing purposes, the staining solution was diluted 1:1 with ethanol. Using this solution, most of the stained peptides can be used for manual Edman degradation. One can make a preliminary identification of the N-terminus of the peptide from the colour of the dyed peptides. Glycine and proline produce a yellow colour, threonine and serine appear as yellow to green spots, tyrosine and histidine are brown, whereas asparagine and glutamine appear with a deep blue colour. With peptides containing lysine, additional colour reaction is obtained from the dye-coupled ε-amino-group. At room temperature, the staining is complete after 24 hr. By increasing the temperature to 40°C, visualization was possible after 5 hr.

The peptides were extracted from the silica gel using acetic acid. The silica gel, containing the fluorescamine or ninhydrin-stained peptides was scraped off the thin layer plate, and the peptides were extracted with 600 µl of 50% acetic acid. After shaking for 10 min. and centrifugation,

the peptides were further extracted with another 400 µl of 50% acetic acid. The extracts were pooled, 700 µl used for Edman degradation and 300 µl for amino acid analysis.

The cyanogen bromide cleavage products were dissolved in 1 ml H_2O and a sample of 10 µl was checked by SDS-slab-gel electrophoresis for composition. The peptides were separated at room temperature on a Sephadex-G-50-column (180 x 1 cm) in the presence of 10% acetic acid. The flow rate was 6.6 ml/h and fractions of 0.8 ml were collected. The void volume was 35 ml. Every second fraction was analysed for the presence of peptides by SDS-slab-gel electrophoresis. Fractions containing the various peptides were pooled and 20% of the volume used for amino acid analysis, the rest being prepared for solid-phase sequencing.

2.13. Thermal denaturation of ribosomes and nucleic acid/protein complexes

Thermal denaturation effects were followed by measuring the hyperchromicity of nucleic acids at 260 nm. All experiments were performed in buffer 2 (c.f. section 2.2.).

Samples of 70S ribosomes in a concentration of 1 A_{260} unit/ml were placed in a quartz cell with a pathlength of 1 cm, sealed with a Teflon stopper and heated as described below.

A constant amount of nucleic acids (1 A_{260} units/ml) was mixed with increasing amounts of protein (1, 2, 4, 8 nmoles) in a final volume of 1.1 ml.

Temperatures and absorbances were recorded directly in a PYE Unicam-Spectrophotometer, connected to a X-Y recorder to draw a temperature-absorbance graph. A thermo-regulated cell compartment was attached to the spectrophotometer to provide continuous heating of the cuvette. The temperature was read by means of a thermo-couple immersed in a reference cuvette. The rate of temperature increase was 0.5°C/min. and the experiment was performed between 40°C and 100°C. The melting temperature was characterized by the inflexion point of the slope of the graph.

2.14. Circular dichroism: thermal and urea denaturation of proteins

The denaturation of proteins was followed by measuring the ellipticity at 220 nm. The CD-Spectra were recorded using a Cary 60 Spectrometer with a cell of 1 mm pathlength. The temperature of the cell compartment was maintained using an external circulating temperature bath. Evaporation was avoided by sealing the cell with a Teflon stopper. Campher sulfonic acid was used for calibration. Proteins at a concentration of 120 µg/ml were dialysed against 350 mM KF, 5 mM potassium phosphate buffer, pH 7. The time constant was 3 and the sensitivity was 0.020/full scale. The baseline was checked before each trial by running a spectrum of pure buffer solution. Thermal denaturation of proteins was performed gradually in steps of 5 degrees. The spectrum was recorded after an equillibration time of 15 min.

Urea denaturing experiments were carried out at 20°C. Samples of equal protein content were mixed with increasing amounts of urea buffer (8 M urea, 350 mM KF, 5 m potassium phosphate buffer, pH 7) and recorded after a period of 1/2 hour. The spectra were interpreted by calculation of the decrease in the α-helix content measured at 220 nm on the basis of formula (1):

$$(1)\quad \theta = \frac{MG \cdot \theta_\lambda}{100 \cdot c \cdot d} \quad \frac{\text{degree}}{\text{cm}^2 \cdot \text{decimol}}$$

MG = mean residue molecular weight; θ_λ = observed ellipticity in degrees; c = concentration in gram/ml; d = pathlength in cm.

2.15. Proton magnetic resonance spectroscopy of proteins (PMR)

The PMR-spectra of proteins were measured on a Bruker WH-270 spectrometer (Biophysics Dept., Polytechnic, Portsmouth, U.K.). Protein samples at a concentration of 3 mg/ml in a volume of 0.3 ml were dialysed against H_2O and then dialysed against 0.1 M NaCl and 10 mM sodiumphosphate, pH 7. Urea denaturation of proteins was performed in the same buffer containing 8 M [^{2}H]urea. The spectra of nucleotide bound proteins were recorded in the presence of adenine/thymidine desoxynucleotides in the molar protein:nucleotide ratio of 1:2. After incubation for 0.5 hr. the samples were centrifuged and transferred into a 5 mm PMR-tube. Spectra were recorded at 20°C in a Bruker WH-270 magnetic resonance spectrometer oper-

ating in a Fourier Transform mode. A pulse length of 12 usec was used and data were collected over 0.5 sec for each pulse. The spectra were recorded over a period of 30 min. - 2 hr. corresponding to 2000 - 10.000 transients. Chemical shifts were measured relative to sodium 2,2, dimethyl-2-silapentane sulfonate (DSS).

2.16. Crystallization of and X-ray analysis of proteins

Crystallization experiments were performed in sealed Linbro immuno trays using the vapour diffusion technique. Solutions of ammonium sulfate and MPD were used as precipitants.

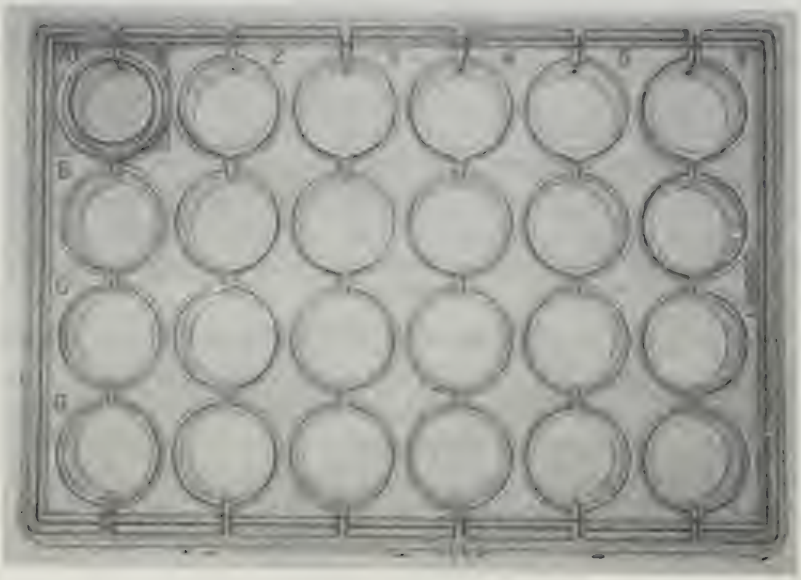


Fig. 6: Linbro immuno tray used in crystallization experiments.

Protein solutions at concentrations of 4 - 30 mg/ml were dialysed against H_2O. 10 ul of the protein solution were mixed with 10 ul of the precipitant. The following precipitant solutions were used:

Solution 1: 4 M $(NH_4)_2SO_4$ (saturated)
1 M Bicine pH 8.8

Solution 2: 4 M $(NH_4)_2SO_4$
1 M Hepes, pH 7.4 (NaOH)

Solution 3: MPD (30% V/V)
100 mM Na_2HPO_4, pH 7.4 (NaOH)

The buffer solutions contained PMSF, BAM and 2-mercaptoethanol in the concentration of 0.1 mM, 0.2 mM and 12 mM, respectively.

The 20 µl droplets were transferred to a microscope cover slip, sealed tightly over a reservoir with silicon grease. The reservoir contained 1 ml of precipitant solution. Crystallization of the proteins was performed by vapour diffusion of the droplets against increasing concentrations of precipitant at 18 - 20°C. Suitable crystals for X-ray analysis were washed with the corresponding crystallization buffer and mounted in thin-walled capillaries.

Screenless procession photographs were recorded at room temperature on a Enraf-Nonius camera. CuKα-X-radiation was provided by a Seifert stationary anode operating with a fine focus tube at 40 KV and 30 mA.

3. Results

3.1. Bacteria

Thermus thermophilus

Th. thermophilus is a Gram-negative thermophile bacterium. It was first described by Oshima and Imahori (60). It is an obligate aerobe and grows optimally between 70°C - 80°C. The maximum temperature for growth is about 85°C and the lowest 48°C.

Strain HB8 was obtained from DMS, Göttingen, FRG. The bacteria were grown at 80°C as well as at 55°C in order to search for possible alterations in the thermostability of ribosomes as well as differences in the 2-dimensional-gel electrophoresis pattern of the ribosomal proteins.

The growth of the cells at 80°C was very dependent on the stability of the fermenter. After a temperature drop from 80°C to 70°C growth stopped, followed by a lag phase of 3 - 4 hr. The generation time was 3.5 hr. Cells grew rapidly up to 0.7 - 0.8 OD_{650}nm. Per fermentation the yield of cells was, on the average, 70 g. The total amount of cells obtained was 1600 g wet weight.

Fermentation of cells at 55°C was performed in the same way as described in Methods (Section 2.1.). A preculture grown at 80°C was incubated over a period of 12 hr. at 55°C with a generation time of 2.5 hr. The preculture, adapted to the new temperature, was used for inoculation of the fermenter and grown as described above. The generation time of the cells grown in the 100 l fermenter was 3.5 - 4 hr., which might be due to the insufficient oxygen supply and mixing. The total yield of cells grown at 55°C was 950 g wet weight (approximately 70 - 90 g per fermenter run).

Thermus aquaticus

The Th. aquaticus is a Gram-negative obligate aerobic thermophile which was first described by Brock and Freeze (47, 61, 95). The organism grows optimally at pH 7.5 to 7.8. The optimal growth temperature is 70°C, the maximum 79°C and the minimum 40°C. Thermus aquaticus cells (strain EP 00276) were obtained from CAMR, Porton Down, U.K.

Bacillus caldolyticus

B. caldolyticus is a Gram-positive thermophilic aerobe which was isolated by Heinen and Heinen (63). The temperature optimum of growth is about 78°C - 80°C (98). The cells (strain Nr. EP 00275) were obtained from CAMR, Porton Down, U.K.

Clostridium pasteurianum

This organism is an obligate anaerobe. It is non-pathogenetic, Gram-positive and able to fix nitrogen. The optimal growth temperature is 37°C (64). Based on the homology between 5S rRNA, C. pasteurianum and B. stearothermophilus appeared to be closely related (5). Therefore, it was of interest to compare the ribosomal protein pattern of the two organisms. The cells (strains NCIB 9486 and EP 0025) were obtained from CAMR, Porton Down, U.K.

3.2. Isolation of 70S ribosomes

Ribosomes were isolated by gel filtration in the presence of 1 M NH_4Cl. The total amount of ribosomes from Th. thermophilus, grown at 80°C, was 89.000 A_{260} units/1 kg wet weight cells, and from cells grown at 55°C, 50.000 A_{260} units/1 kg wet weight. Figure 7 shows the SDS-slab-gel electrophoresis pattern of fractions from the Sephacryl-S-300-column. The ribosomes eluted after 3 - 4.25 l, corresponding to fraction 2 - 7.

Th. aquaticus yields a total amount of ribosomes of 34.000 A_{260} units/1 kg wet weight. The low yield of ribosomes is understandable since the cells were harvested at the stationary phase. The elution profile of the S-300-column as analysed on the SDS-gel can be seen in Figure 8.

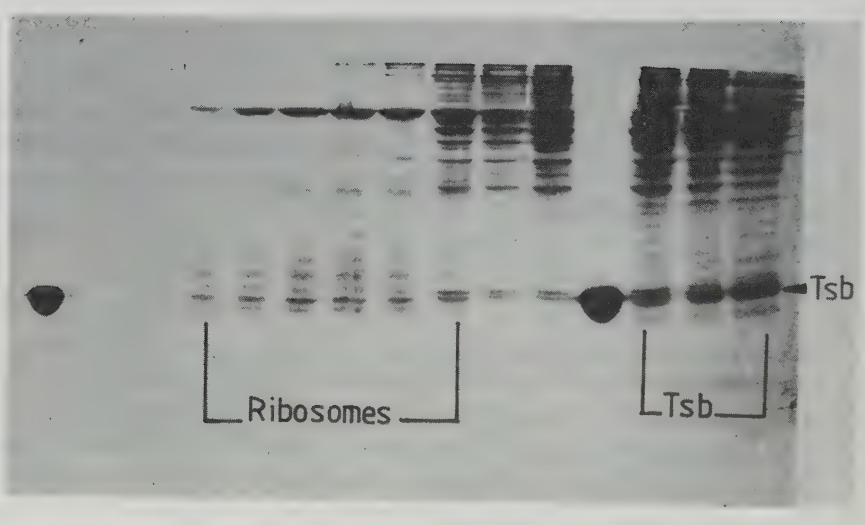


Fig. 7: Ribosomes of Th. thermophilus elute after 3.0 - 4.26 l from the S-300-column.

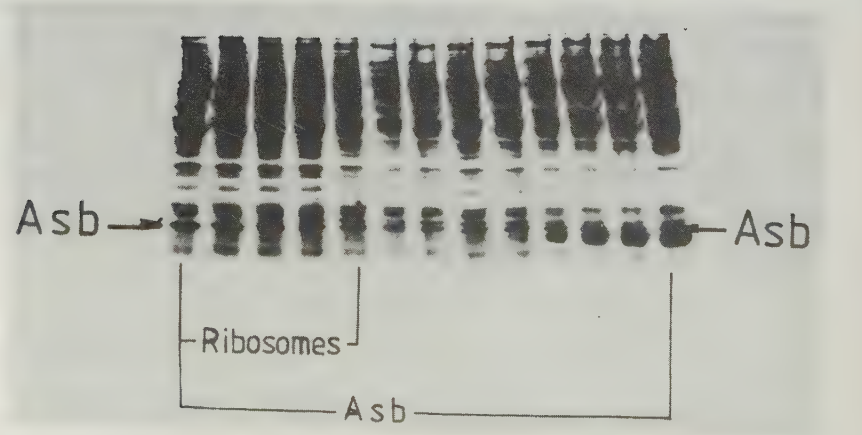


Fig. 8: The Th. aquaticus ribosomes elute at the same position as seen in Figure 7.

The amount of ribosomes obtained from Bacillus caldolyticus was 90.000 A_{260} units/ 1 kg wet weight cells. Figure 9 shows the elution profile of ribosomes from the S-300-column.

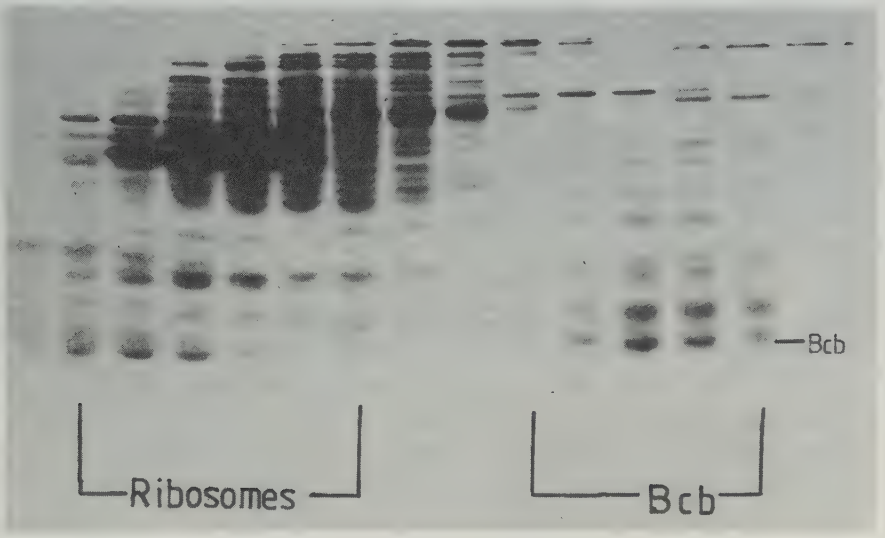


Fig. 9: B. caldolyticus ribosomes from the Sephacryl-S-300-column.

The ribosomes from C. pasteurianum eluted at the same volume as ribosomes from B. caldolyticus. The total amount of ribosomes obtained from 1 kg cells was 42.000 A_{260} units.

3.3. Structural studies on ribosomes and total r-protein extract

3.3.1. Thermostability of 70S ribosomes from Th. thermophilus, grown at 80°C and 55°C.

Figure 10 illustrates the effect of increasing the temperature on the stability of intact ribosomes. Ribosomes grown at 80°C are stable up to 81°C and they possess a Tm of 89°C. This result is in satisfactory agreement with the maximum of growth temperature. The melting curve is highly cooperative and monophasic. The same melting behaviour has been reported

for ribosomes from Th. aquaticus (62) which exhibit a strong similarity to Th. thermophilus ribosomes.

Ribosomes from cells grown at 55°C exhibit a different melting profile. The melting starts very early at 50°C which actually is 5 degrees below the growing temperature. Melting continues to the transition temperature of 80°C. The "premelting" of the ribosomes might be due to heterogeneous population of 80°C and 55°C ribosomes, which is expressed by the gradual increase of the absorbance. On the other hand, it should be mentioned that part of the change could be generated by action of RNase on ribosomes, which was activated by the temperature increase.

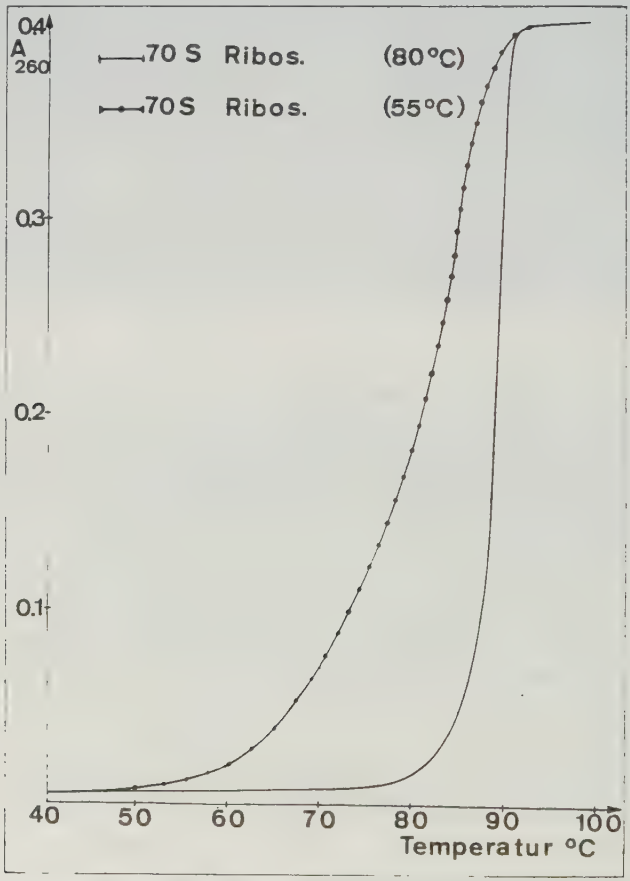


Fig. 10: Melting curve of 70S ribosomes grown at 80°C and 55°C. The absorbance was plotted continuously against temperature. Ribosomes demonstrate a melting temperature of 89°C (grown at 80°C) and 80°C (grown at 55°C).

3.3.2. Two-dimensional-gel electrophoresis of the total protein extract (TP) from subunits and ribosomes

Ribosomes were separated into 30S and 50S subunits by zonal centrifugation. Ribosomal proteins were extracted with acetic acid/urea and the proteins were analysed by two-dimensional-gel electrophoresis (Section 2.11.1.). The proteins were numbered using a tentative nomenclature according to the Kaltschmidt and Wittmann procedure (30). This nomenclature describes the migration of the proteins on the gel and does not imply a sequence homology to "L" or "S" proteins of E. coli. Only protein spots were considered which could be seen on gels of two different extractions, namely with acetic acid, as well as with a combination of acetic acid/urea.

Figure 11 shows the pattern of TP30 and TP50 and TP70 from Th. thermophilus. Even though the ribosomes have been extracted in the presence of 1 M NH_4Cl a few non-ribosomal proteins, exhibiting a high molecular weight, remain attached to the ribosomes. These proteins can be seen in the 50S as well as in the 30S pattern. The number of ribosomal protein spots of the large subunit is about 35 and of the small one about 21 proteins. The number didn't change when two different extraction procedures were used.

Especially interesting is protein 30 from the large subunit, which co-migrates with the Tsb-protein (see below). This protein is attached to the ribosome and can be removed by high salt wash.

Figure 12 shows the 2-D-gel pattern of ribosomal proteins from Th. aquaticus. 24 proteins were extracted from the 30S subunit and 37 proteins from the 50S subunit. In both 30S and 50S subunits, as well as in ribosomes, an interesting protein spot was observed. This protein, assigned as AX appears at almost the same position as the DNA binding protein II from Bacillus stearothermophilus and E. coli (48). The intensity of the stained spot in the 50S subunit is at least 3 - 4 times stronger than the one in 30S. It is likely that even in the presence of high salt concentrations not all non-ribosomal proteins were removed from the ribosome.

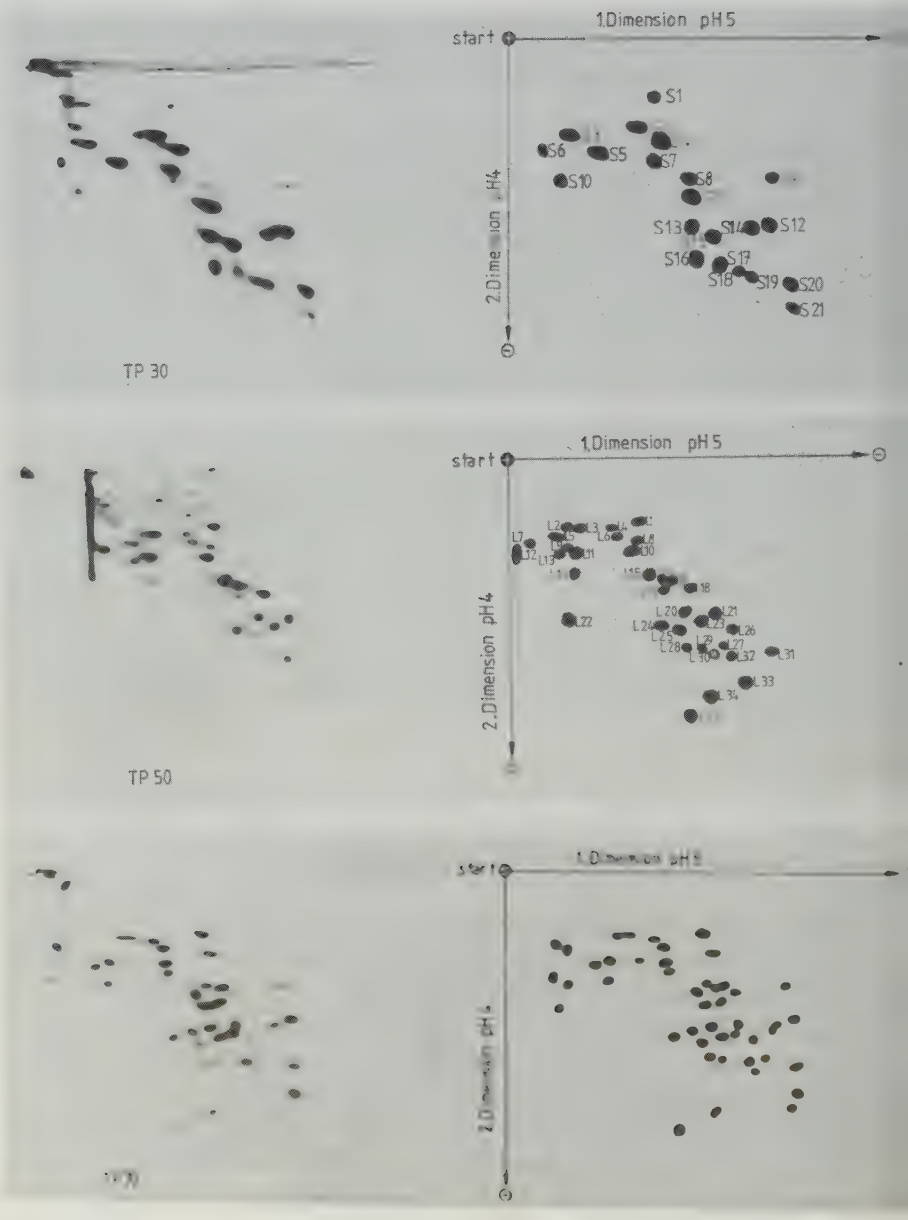


Fig. 11 Th. thermophilus, two-dimensional-gel electrophoresis of 30S, 50S and 70S proteins. A schematic diagram of the gels is presented on the right side. The spots have been given a tentative number.

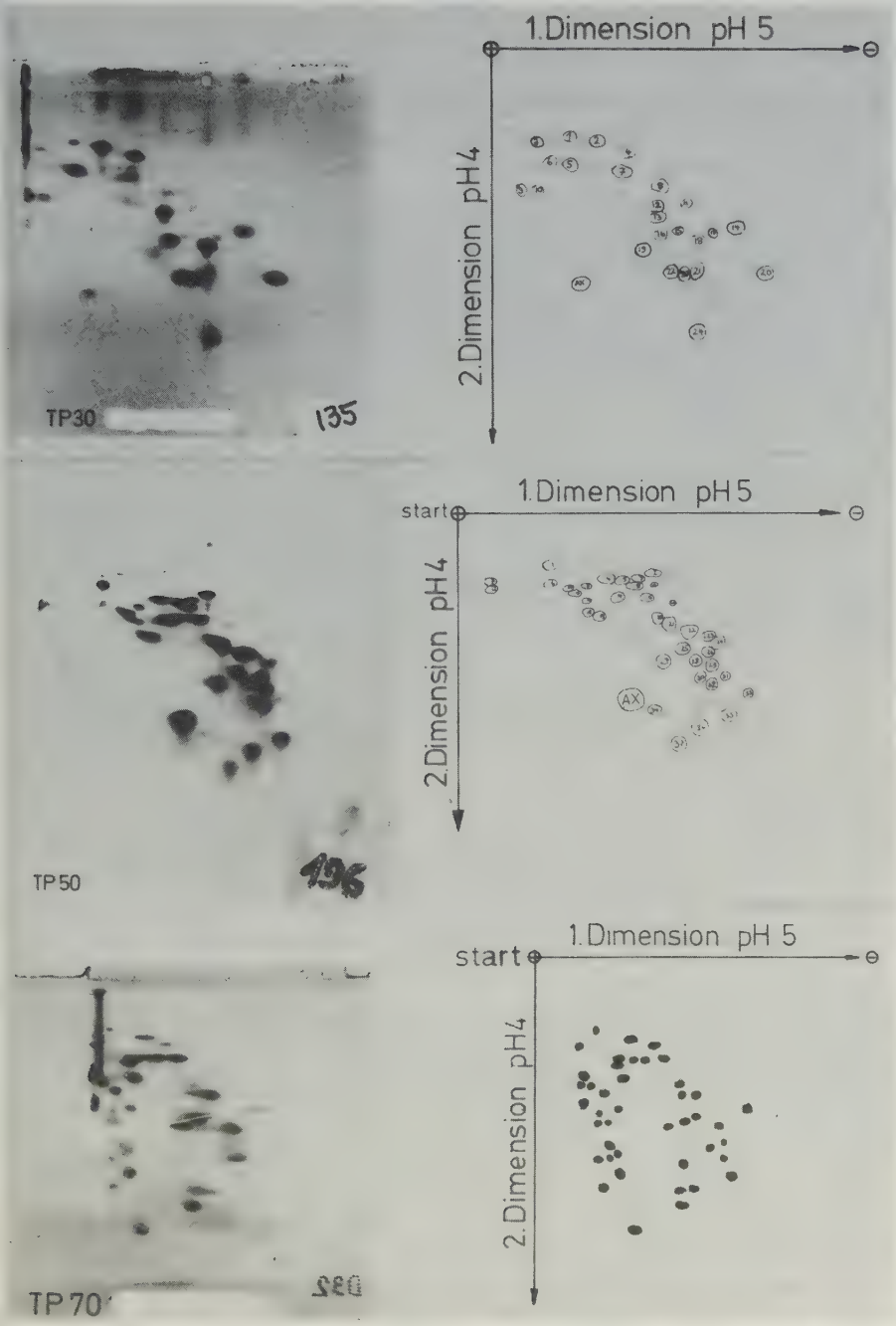


Fig. 12: Total protein extract from subunits and ribosomes from Th. aquaticus. On the righthand side, the schematic diagram.

When comparing the two-dimensional-gel pattern of ribosomal proteins from Th. aquaticus and Th. thermophilus, similarities in the number as well as in the electrophoretic mobility can be seen. Figure 13 shows the co-migration pattern of 30S and 50S proteins from the two bacteria.

Fig. 13: Th. aquaticus/Th. thermophilus co-migration pattern of proteins from the subunits. (A). 30S proteins Th. aquaticus; (B). co-migration of 30S proteins Th. aquaticus and Th. thermophilus; (C). 30S proteins Th. thermophilus; (D). 50S proteins Th. aquaticus; (E). co-migration of 50S proteins Th. aquaticus and Th. thermophilus; (F). 50S proteins Th. thermophilus.

The proteins from the two bacteria are mostly basic and exhibit similarities in molecular weight. Small differences can be seen on the charge of proteins which migrate at the same position of the molecular weight.

The total protein extracts of the 30S as well as 50S subunit from Bacillus caldolyticus and Bacillus stearothermophilus were also compared by 2-D-gel electrophoresis as shown in Figure 14. The electrophoresis reveals a strong similarity on the r-proteins from both organisms. Small differences can be seen in the 50S pattern of B. caldolyticus, since there are 2 additional protein spots (marked with an arrow). This could be due to a washing artefact of ribosomes, as it was shown for the Tsb- and AX-protein.

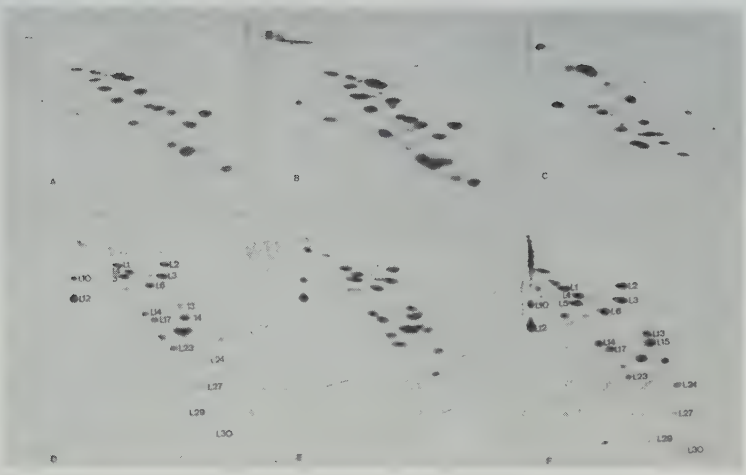


Fig. 14: B. caldolyticus/B. stearothermophilus co-migration pattern of proteins from the subunits. (A.) 30S proteins, B. caldolyticus; (B.) 30S protein from B. caldolyticus/B. stearothermophilus, co-migration; (C.) 30S proteins, B. stearothermophilus; (D.) 50S proteins, B. caldolyticus; (E.) 50S proteins, co-migration pattern; (F.) 50S proteins, B. stearothermophilus.

Figure 15 shows the 2-D pattern of ribosomal proteins from C. pasteurianum. 25 protein spots are seen in the small subunits and 32 in the 50S subunit. The ribosomal proteins from B. caldolyticus and C. pasteurianum show a high degree of similarity, in particular in the central region of the 30S and 50S pattern.

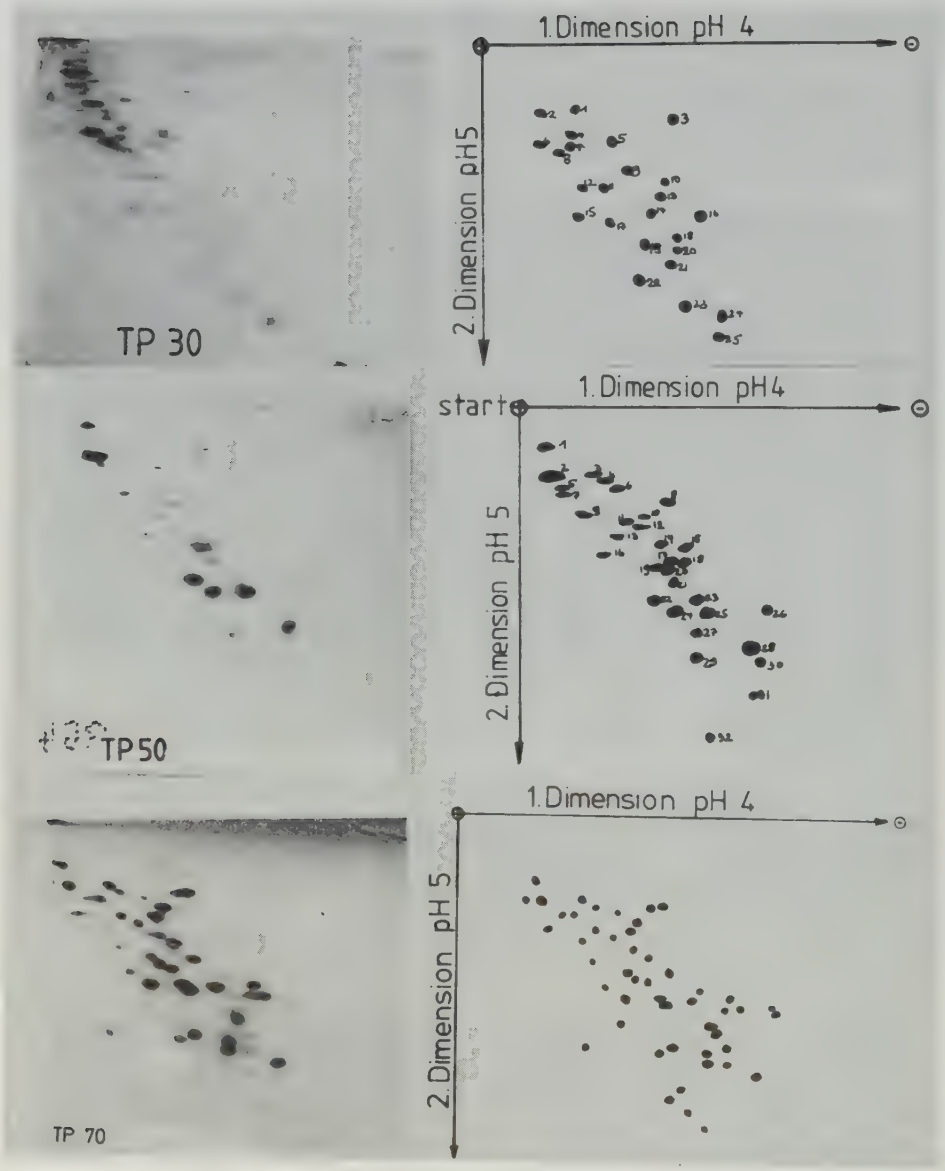


Fig. 15: Total protein extract from subunits as well as ribosomes from Clostridium pasteurianum. On the right hand side, the schematic drawing.

3.4. Isolation of DNA binding proteins

These proteins were found in the fractions eluting after the ribosomal peak from the Sephacryl-S-300-column (elution volume 5 - 6 l, corresponding to fraction 12 - 16). They were purified by ionic exchange chromatography, affinity chromatography and gel filtration.

The DNA binding protein designated Tsb-protein from Th. thermophilus, grown at 80°C was eluted from a CM-Sephadex-C-25-column at a NaCl concentration of 550 mM. The protein was purified on a Heparin-Sepharose-Cl-6B-column and eluted at the same NaCl concentration as before (550 mM).

The total amount of Tsb obtained from 1 kg cells was approximately 30 mg.

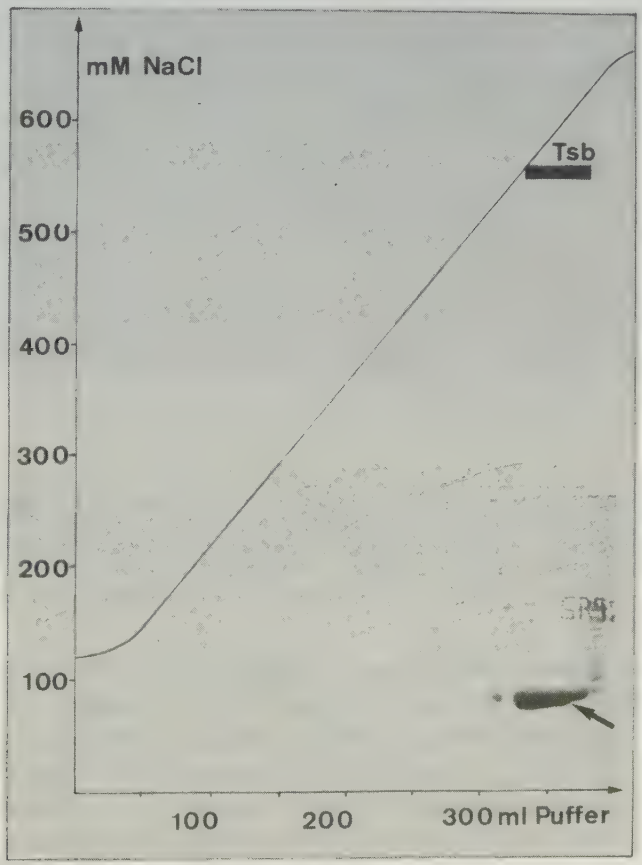


Fig. 16: Tsb-protein purification on a CM-Sephadex-C-25-column with 550 ml 0.0 M - 1.0 M NaCl linear gradient in 20 mM sodium phosphate buffer, pH 7. Tsb elutes at 550 mM NaCl.

The corresponding Tsb-protein was not found during preparation of ribosomes from cells grown at 55°C. Instead, two proteins exhibiting a lower molecular weight were found in the S-300 fractions. Further purification on a CM-Sephadex-C-25-column, followed by Heparin-Sepharose-chromatography (even in the presence of 6 M urea), revealed a protein mixture which could not be separated. Considering the small amount of protein obtained (approximately 500 µg), as well as the blocked N-terminus, futher investigations were stopped.

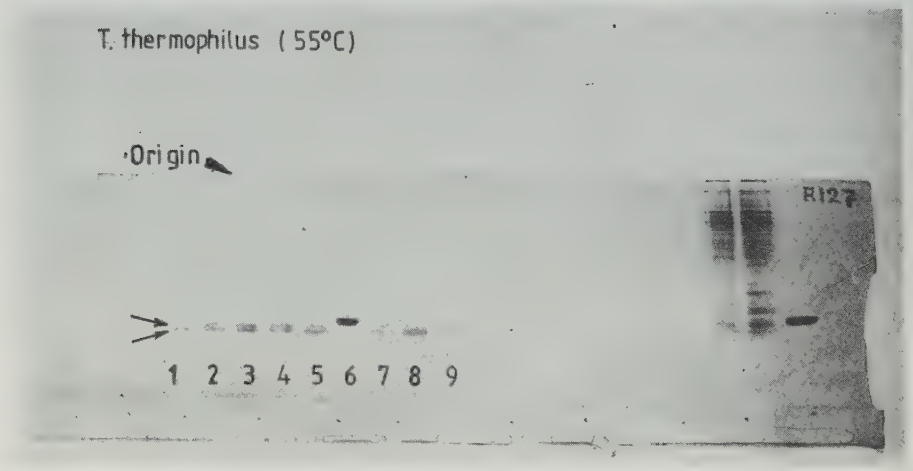


Fig. 17: Purification of DNA binding protein from Th. thermophilus, grown at 55°C. Two small proteins were found in the S-300 fractions and purified on a Heparin-Sepharose-Cl-6B-column in the presence of 6 M urea. The mixture elutes at 500 mM NaCl. Lanes 1 - 5 and 7 - 9 show the two proteins, lane 6 is Tsb.

Most of the binding protein from Th. aquaticus (Asb) was bound to the ribosome (see Fig. 8, fractions 2 - 6 and 11 - 14), therefore, only a small amount of this protein could be obtained from the post-ribosome fractions of the S-300-column. Asb-protein, contaminated with other proteins, eluted from a CM-Sephadex-C-25 ionic exchanger at a NaCl concentration of 200 - 250 mM which required a further purification step on a Heparin-Sepharose-Cl-6B-column and on a Sephacryl-S-200-column. During dialysis against 350 mM NaCl most of the protein precipitated. Since the amount of

Asb-protein was low, purification under non-denaturing conditions for secondary, as well as tertiary structural studies was not reasonable. Therefore, we decided to purify the small part of the protein for primary structure studies in the presence of 20% acetic acid.

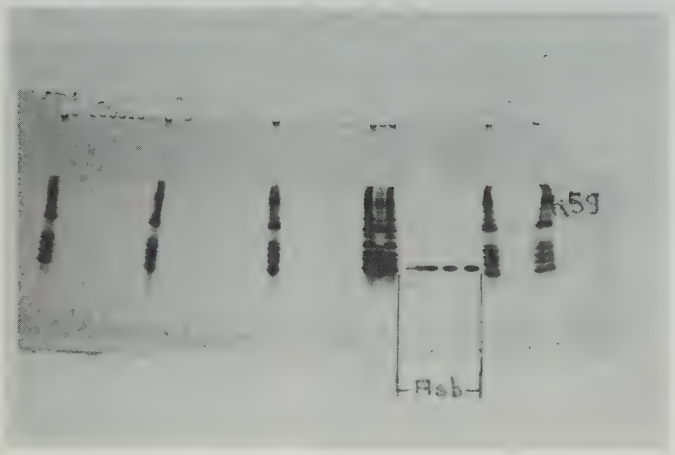


Fig. 18: Asb-purification on a S-200-column.

Asb-protein eluted from the Sephacryl-S-200-column at 1180 - 1280 ml. The protein was concentrated by TCA-precipitation, dissolved in 10% acetic acid and stored frozen. The total yield of Asb-protein was 300 µg.

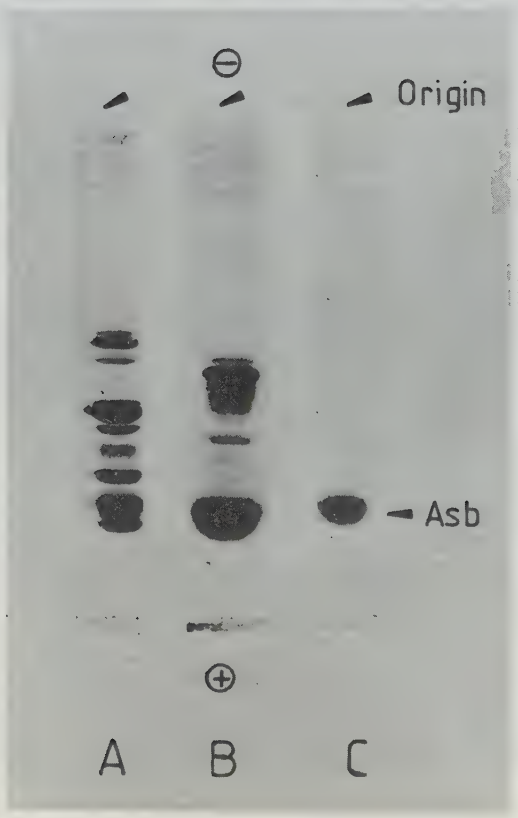


Fig. 19: Asb-protein elutes together with several high molecular weight proteins. (A) Asb-fraction from the CM-Sephadex-C-25-column; (B) Asb-fraction from the Heparin-Sepharose-Cl-6B-column; (C) Asb-fraction from the Sephacryl-S-200-column.

The DNA binding protein of B. caldolyticus, Bcb, (see Fig. 9, fractions 12 - 14), eluted from a CM-Sephadex-C-25-column at 250 - 300 mM NaCl. A similar purification was performed on a Heparin-Sepharose-Cl-6B-column. The protein eluted at the same NaCl concentration. The total yield of Bcb-protein was approximately 60 mg/1 kg cells wet weight.

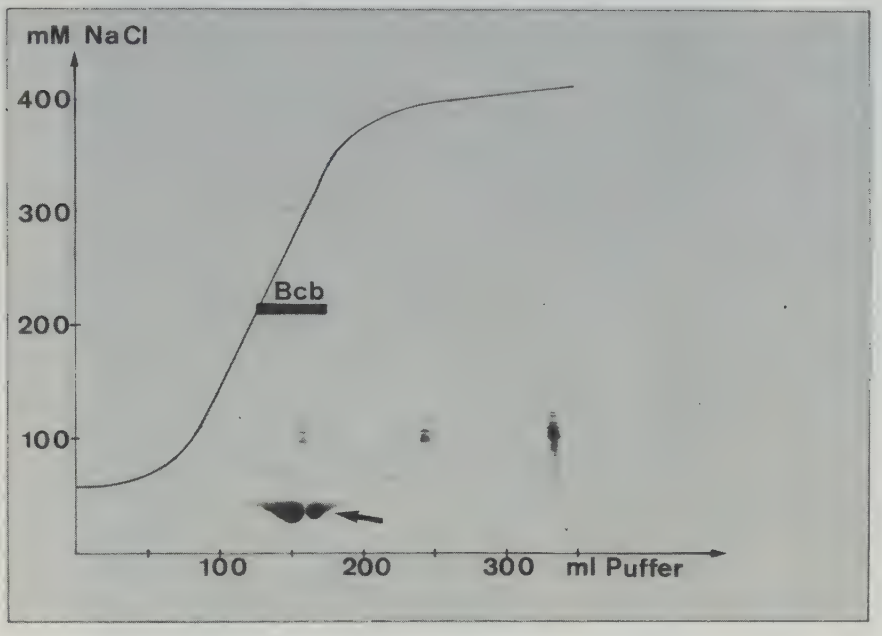


Fig. 20: Bcb-protein elutes from a Heparin-Sepharose-Cl-6B-column at 250 - 300 mM NaCl.

Fractions of the Sephacryl-S-300-column containing the Csb-protein were applied to a CM-Sephadex-C-25-column. The DNA binding protein eluted as a broad peak in the presence of several other proteins at 200 - 400 mM NaCl (Fig. 21).

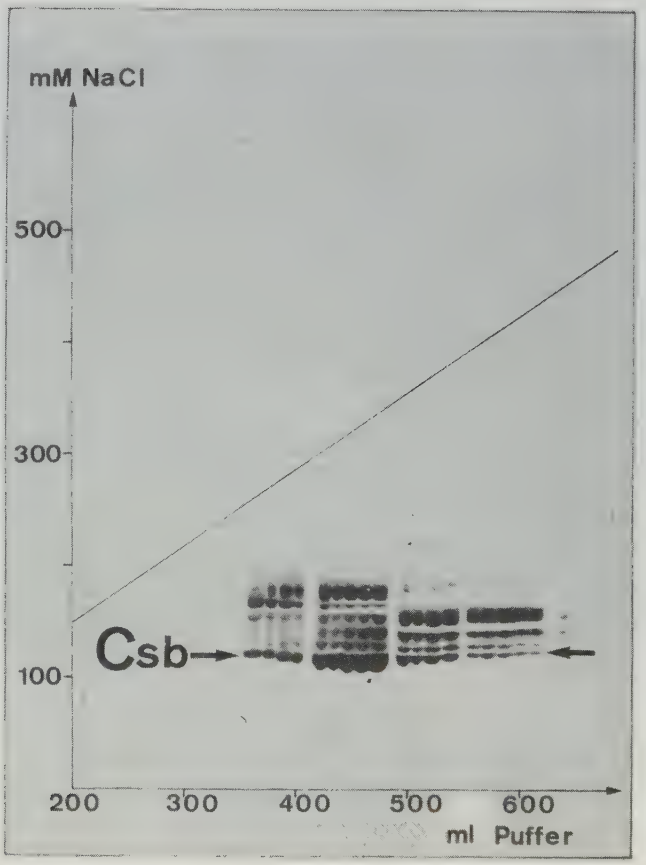


Fig. 21: Fractions from the Sephacryl-S-300-column, containing the Csb-protein, were loaded on a CM-Sephadex-C-25-column. Csb eluted in the presence of several proteins of a higher molecular weight at 200 - 400 mM NaCl.

Since the contaminations exhibit differences in their molecular weight, as well as in their charge, fractions containing the Csb-protein were divided into 2 parts for separate purification on gel filtration and affinity chromatography. Fractions with high molecular weight impurities (fraction volume 350 - 500 ml, Fig. 20) were applied to a G-50-column. Pure Csb-protein eluted after 1180 ml.

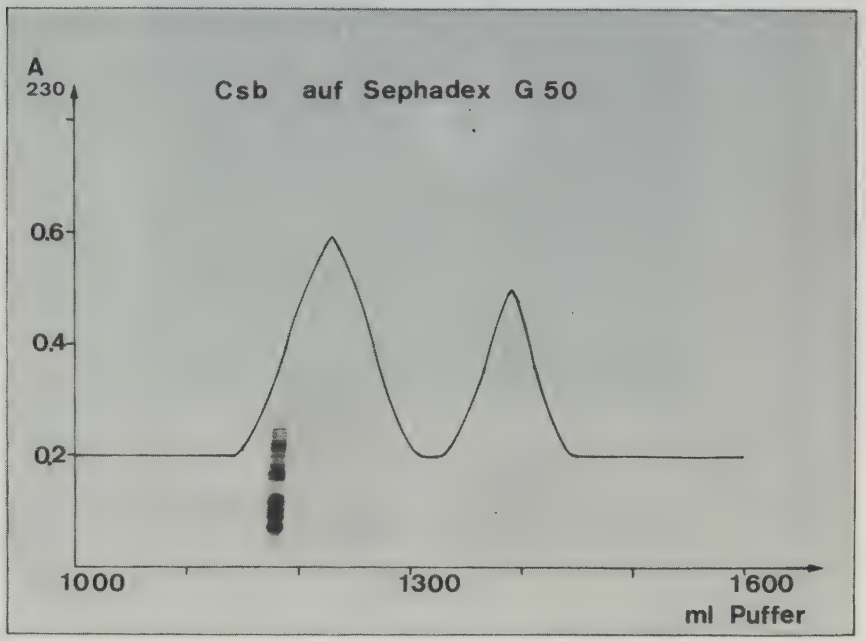


Fig. 22: Purification of the Csb-protein. Contaminations exhibiting a high molecular weight were separated on a G-50-column. Pure Csb was contained in the first peak.

Fractions with low molecular weight impurities (fraction volume 50 - 630 ml, Fig. 21) were applied on a Heparin-Sepharose-Cl-6B-column and pure Csb-protein eluted at 350 mM NaCl.

The total yield of Csb-protein, obtained from 1 kg cells, was approximately 25 mg.

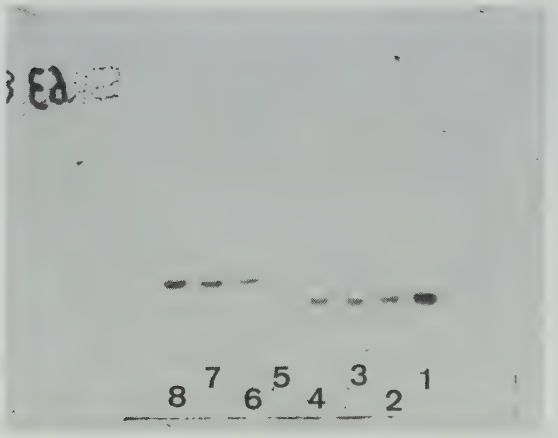


Fig. 23: Csb-protein elutes from a Heparin-Sepharose-Cl-6B-column at 350 mM NaCl. Pure Csb in lanes 1 - 4.

Concentration, determination of the protein concentration and storage of the individual protein were performed as described in 2.5.

3.5. Confirmation of the DNA binding character of the isolated protein

The ability of the proteins to bind to DNA was demonstrated using a filter binding assay and by gel filtration of nucleic acid/protein complexes.

3.5.1. DNA filter binding assay

Increasing amounts of protein were incubated with a constant amount of [^{3}H]DNA (section 2.9.1.) and the DNA retained on the filter was determined by continuous scintillation. The protein/nucleic acid ratios were expressed in weight/weight.

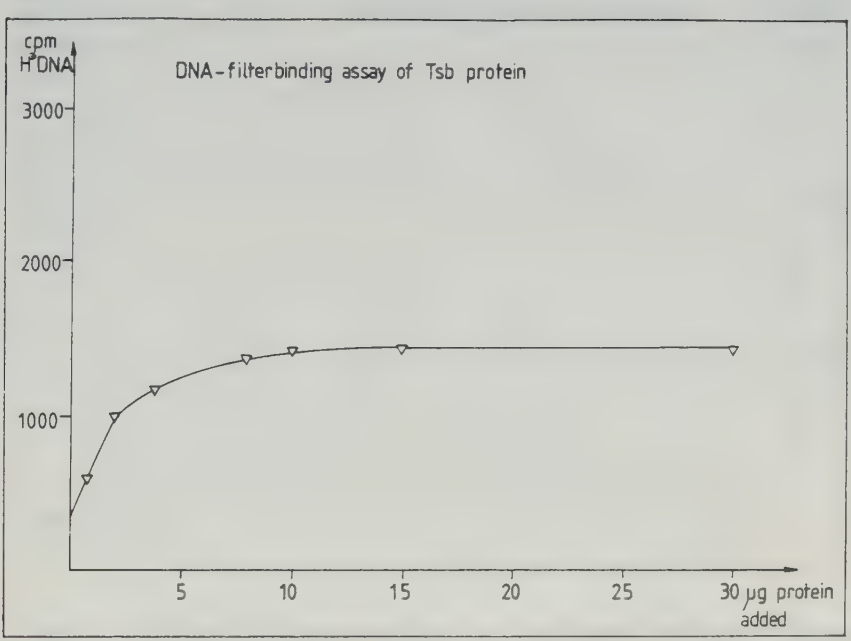


Fig. 24: 1 µg [^{3}H]DNA (2700 cpm) was incubated with increasing amounts of Tsb-protein for 20 min. at 37°C. The nucleo protein complex was filtered through a Millipore filter and counted.

At a ten-fold excess of Tsb a plateau was reached in the DNA binding curve. The binding proteins from B. caldolyticus (Bcb-protein: DNA) and C. pasteurianum (Csb) gave a plateau already at a ratio of 2:1.

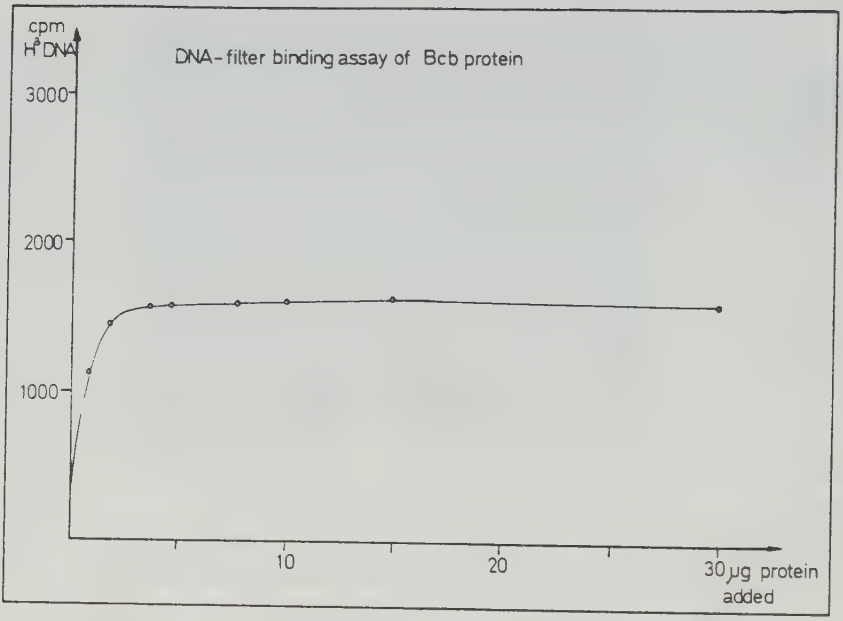


Fig. 25: 1 µg [^{3}H]DNA (2700 cpm) was incubated with increasing amounts of Bcb-protein.

To exclude artificial binding of proteins, a small basic protein, lyzozym, was incubated with DNA and rRNA under the ionic conditions used for the assay of the isolated proteins. No complex formation was observed, neither on the filter assay nor on gel filtration (see below).

3.5.2. Gel filtration of protein/nucleic acid complexes

To confirm the nucleic acid binding affinity of the isolated proteins, two different gel filtration systems at two different flow rates were employed. Each experiment was carried out twice in order to exclude mistakes.

3.5.2.1. Sephadex-G-75 assay

The Tsb-, Bcb- and Csb-protein eluted together with 5S rRNA at 15 ml and were excluded with DNA at 13 ml at a flow rate of 63 ml/hr. At a protein/nucleic acid ratio of 4:1, unbound protein was eluted at the elution volume of the individual binding protein (18 ml).

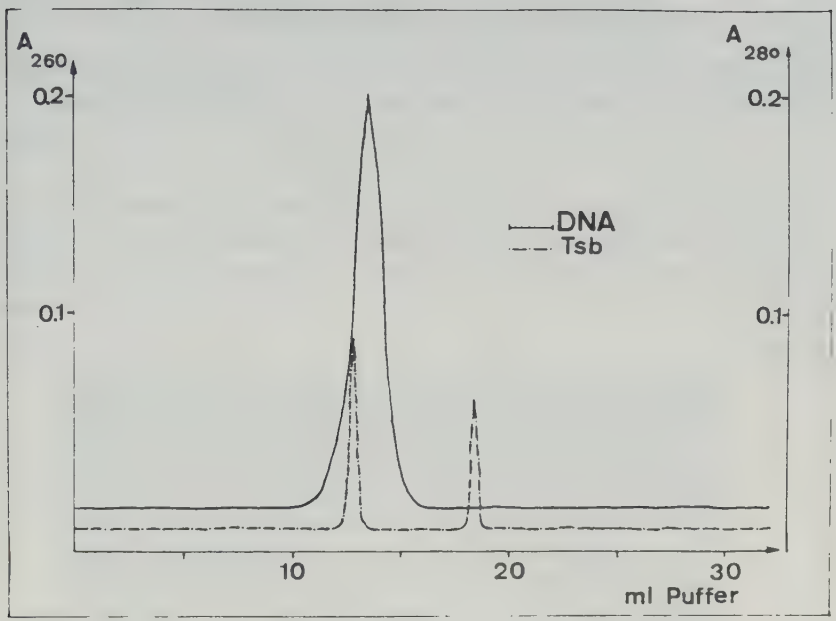


Fig. 26: DNA and Tsb in a ratio of 1:4 (W/W) were incubated and loaded on a G-75-column. Unbound protein elutes at the elution volume of Tsb (flow rate: 63 ml/hr).

By slowing down the flow rate from 63 ml/hr. to 32 ml/hr., the peak of unbound protein increased slightly and the peak of protein bound to nucleic acids was broadened. The protein fraction eluted from 15 to 21 ml (rRNA experiment).

3.5.2.2. Sephadex-G-50 assay

The complex formation was carried out as above and in addition with polycytidylic acid (MG 40.000), the subunits and 70S ribosomes of Th. thermophilus, B. stearothermophilus, B. caldolyticus and C. pasteurianum.

Complex formation could be detected with all tested nucleic acids and ribosomal subunits, as well as with ribosomes. The molar ratio of rRNA-protein in the mixture was 1:2. Excess protein was found at the elution volume of the individual protein (29 - 32 ml). The 70S ribosomes were saturated with Tsb-protein at a molar ratio of 1:40 (ribosome:Tsb). A further increase of the protein concentration resulted in precipitation of ribosomes.

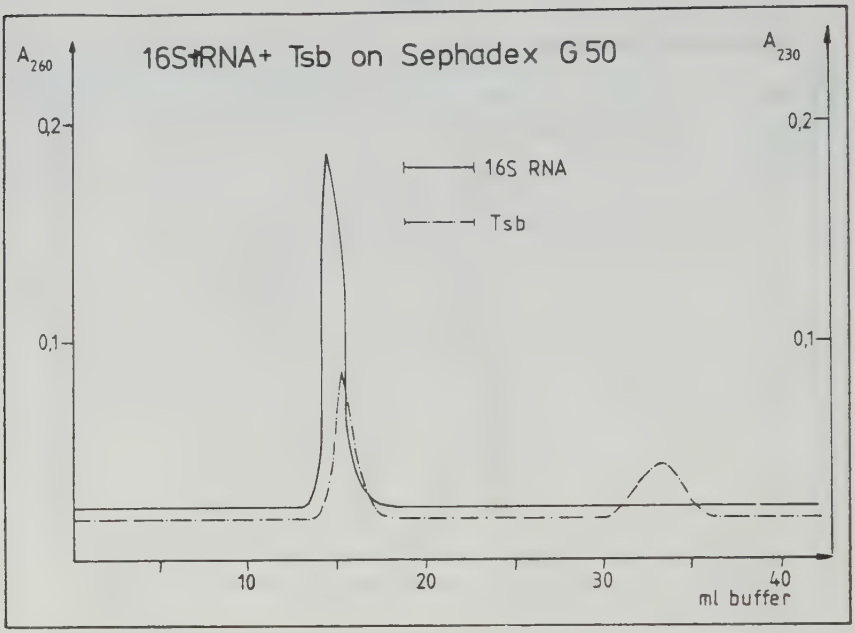


Fig. 27: 16S rRNA and Tsb in a molar ratio of 1:4. The mixture was loaded on a G-50-column, unbound protein elutes at the retention volume of pure Tsb. The flow rate was 88 ml/hr.

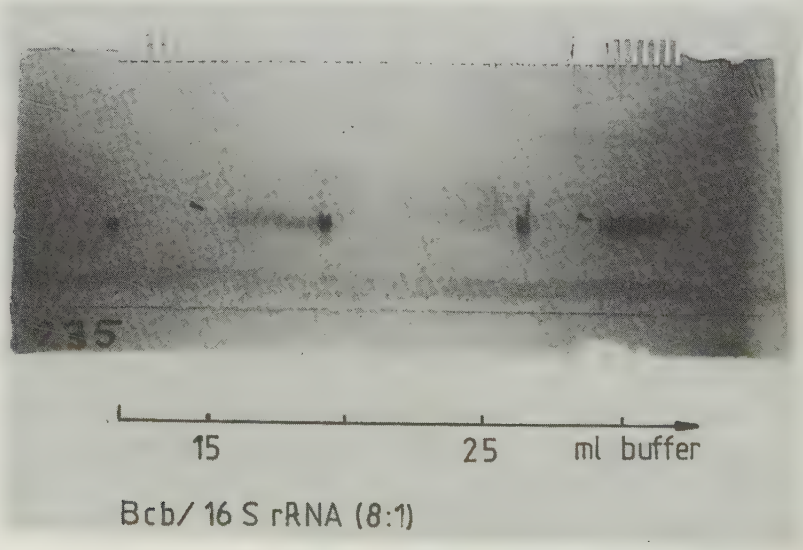


Fig. 28: 16S rRNA and Bcb-protein in a molar ratio of 1:8 on a SDS-slab-gel. Fractions were from the Sephadex-G-50-column.

3.5.3. Electron microscopy of protein-DNA-complexes

Complex formation was performed as described in Section 2.10. The circular plasmid DNA RSF-1010 was complexed with a ten-fold excess of protein (W/W).

The DNA protein complexes of the three individual proteins can be seen in Figure 29. The formation of garland-like DNA structures and the aggregation of protein is a feature which has been observed with DNA binding proteins of E. coli (24, 65 - 67).

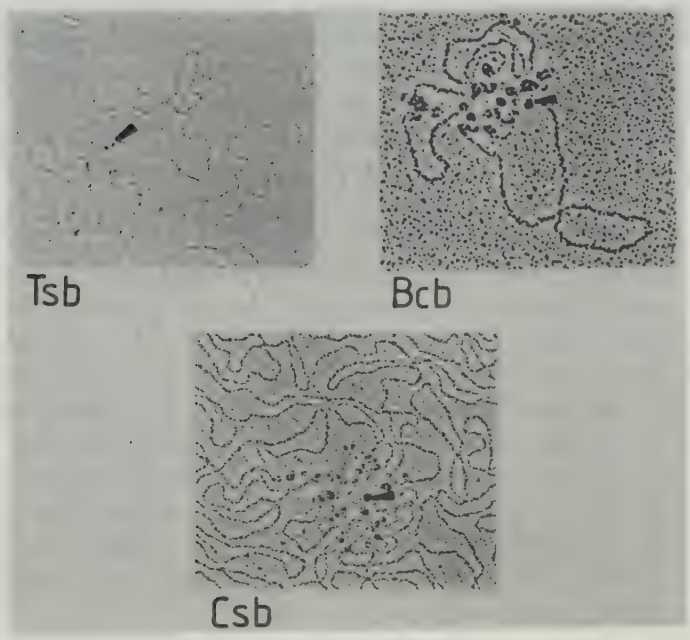


Fig. 29: Tsb-, Bcb- and Csb-protein complex with plasmid DNA RSF-1010. Arrows show protein aggregates

3.5.4. Thermal denaturation of protein/nucleic acid complexes

Melting experiments were performed with Tsb-proteins in the presence of 5S rRNA and DNA from Th. thermophilus. Complex formation of nucleic acids and Tsb was carried out as described in Section 2.13. Changes of the hyperchromicity upon heating, which indicate an alteration of the sec-

ondary structure of the nucleic acid, were recorded continuously and plotted versus temperature.

Figure 30 shows the melting profile of pure 5S rRNA and 5S rRNA in the presence of increasing amounts of Tsb. Pure 5S rRNA starts to melt at 50°C and has a transition temperature of 81°C. At 93°C the absorbance has reached 30%, indicating complete melting of the rRNA structure. The smooth and gradual increase of the absorbance in the temperature range between 65°C and 90°C should reflect a relaxation of unpaired bases followed by a helix-coil-transition of double-stranded sections of the molecules.

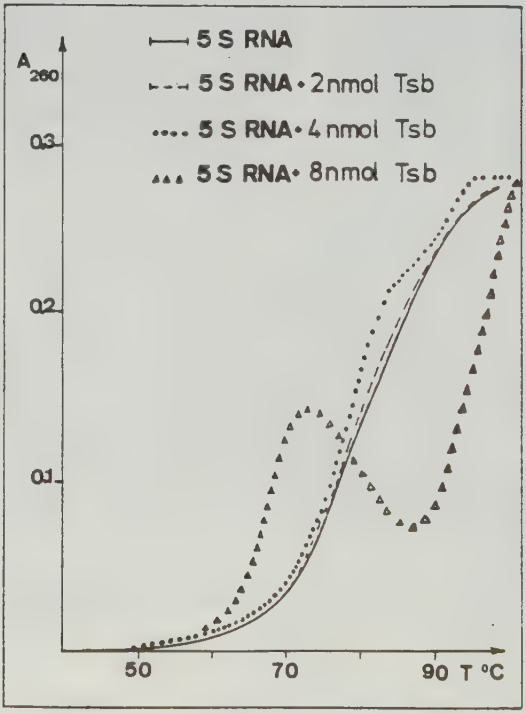


Fig. 30: Melting curve for 5S rRNA and Tsb-protein from Th. thermophilus. The RNA was temperature denatured in the presence of increasing amounts of Tsb protein.

In the presence of 4 nmoles Tsb, the thermal denaturation profile becomes slightly biphasic. A smooth and gradual increase of the absorbance up to 70°C is followed by a rise of the slope up to 82°C which is accompanied by a sharp melting point at 77°C. Between 82°C and 87°C the slope of the profile resembles the curve between 65°C and 70°C. The smooth and gradual increase is followed by another rise of the slope of the absorbance between 87°C and 93°C, which is characterized by a second comparative transition point at 87°C.

The addition of 8 nmoles Tsb provide a complete change of the melting profile. The melting curve had a pronounced biphasic character, but there is no smooth and gradually premelting as could be seen with lower protein concentrations at the complex. Melting starts at 50°C with a rise of the slope of the absorbance at 60°C, reflecting a highly cooparative transition which is indicated by a sharp melting point at 67°C. At 73°C the absorbance has increased to a maximum of 0.14 A_{260}, which is followed by a diminution to a minimal value of 0.06 A_{260} at 87°C. Further increase of the temperature leads to a strong rise of the slope of the absorbance, demonstrating a sharp, cooperative melting point at 95°C. It seems that Tsb-protein decreases as well as increases the transition temperature of 5S rRNA. At a molar rRNA:protein ratio higher than 1:4, base stacking of unpaired rRNA sections might be stronger loosened by the Tsb-protein which is reflected by lowering of the first transition point. By increasing the temperature, this loosened part of the rRNA molecule might be liberated from the "destabilizing" protein and the rRNA might proceed to another conformation. The Tsb-protein undergoes thermal denaturation at 85°C, therefore it should be possible that the polypeptide chain acts like a polycation which stabilize the base-paired sections of rRNA. This complex formation should be responsible for the conformational change of the rRNA at 87°C. Further increase of the temperature leads to a dissociation of protein-rRNA molecules and therefore to denaturation of the individual compounds which is reflected by a highly cooperative rRNA melting at 99°C.

Figure 31 illustrates the melting profiles of pure DNA and DNA in the presence of increasing amounts of Tsb-protein. Pure DNA is stable up to 75°C. Increasing the temperature to 80°C, the DNA starts to melt gradually. The Tm can be seen at 87°C. The addition of 1 nmole Tsb reveals no change of the melting profile. The helix-coil transition is shifted to 88°C and appears to be more cooperative.

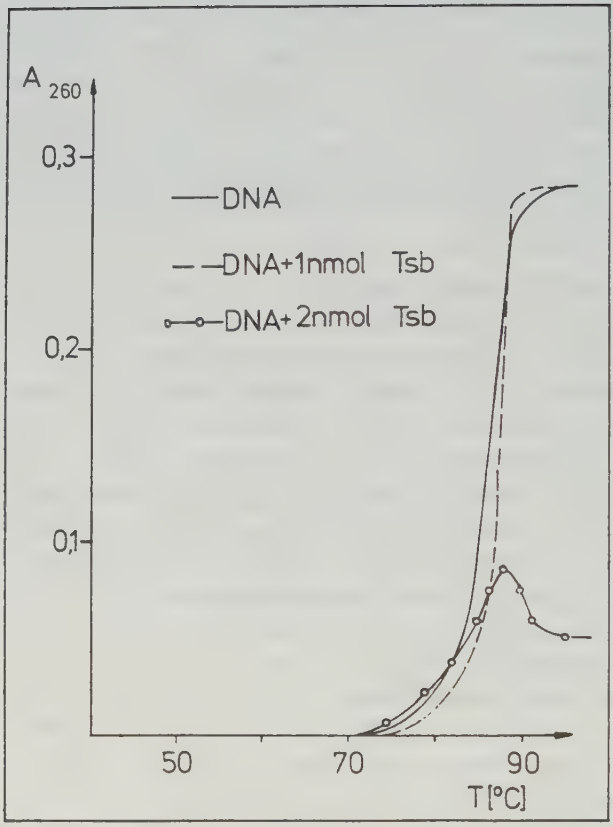


Fig. 31: DNA and Tsb from Th. thermophilus. The protein causes a precipitation of DNA at the transition temperature of DNA.

In the presence of 2 nmole Tsb, the melting profile is changed. The melting starts at 78°C and the helix-coil transition is characterized by the half of the maximal absorbance at 82°C. The maximal absorbance can be seen at 88°C (0.09 A_{260}). Further increase of the temperature is reflected by a diminution of the absorption to a plateau at 92° which is caused by the precipitation of the complex. The same profile is obtained in the presence of 4, 6 and 8 nmole Tsb, respectively. The precipitation of the complex always appears at 88 - 90°C. The protein is supposed to denature the DNA as well as the rRNA at low, as well as high, concentrations.

3.6. Structural studies on the isolated proteins

The investigation of the structure of the isolated proteins was performed on the primary, secondary and tertiary structure level.

Two-dimensional-gel electrophoresis was made on the individual proteins to compare the migration with similar proteins from E. coli and B. stearothermophilus. The migration pattern reveals the similarity of homologous proteins. The N-terminal sequence determination and enzymic digestion of the proteins show identical residues in parts of the primary structure and also show the relationships between the individual proteins.

Secondary structure studies, namely physico-chemical stability features, were investigated by circular dichroism spectroscopy. The diminution of the α-helical amount of the proteins was followed under the influence of thermal and other denaturing means, such as urea. The dependence of the helix-random-coil transition of the proteins on the maximum temperature of growth of the bacteria concerned will be shown.

The tertiary structure was studied on proton magnetic resonance spectroscopy (PMR) and crystallization of the proteins followed by X-ray analysis.

3.6.1. Two-dimensional-gel electrophoresis of the DNA binding protein

The 2-D-gel electrophoresis was carried out as described (Section 2.11.2.). The co-migration pattern of 50S proteins from Th. thermophilus and Tsb-protein can be seen in Figure 32. Tsb appears at the very basic, low molecular weight part of the pattern. The migration features concerning the first dimension are completely different from the binding proteins of E. coli (NS) and B. stearothermophilus (Bsb). Those proteins can be seen in the lower basic part, but at similar molecular weight values. To improve the resolution of the protein spot on the 2-D-gel pattern, decreasing amounts of Tsb were loaded on the gel while the concentration of the 50S-proteins was constant. By this procedure it could be revealed that Tsb co-migrates together with protein L30. On the other hand, it cannot be excluded that protein L30 is a contamination of the ribosome corresponding to Tsb which was not removed by 1 M NH_4Cl wash.

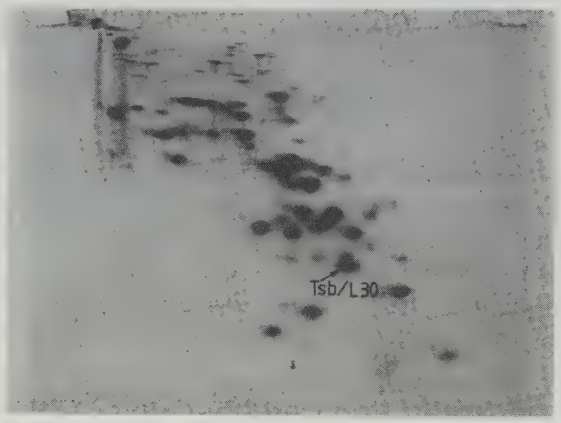


Fig. 32: Tsb-protein and 50S-proteins from Th. thermophilus. Tsb co-migrates with protein L30.

Figure 33 shows the co-migration pattern of 50S-proteins from Th. aquaticus and Asb-protein. The protein co-migrates together with the AX-protein. Most likely, the Asb-protein was not completely removed from the ribosomes by column chromatography and therefore occurs in the protein extract of ribosomes as well as on subunits.

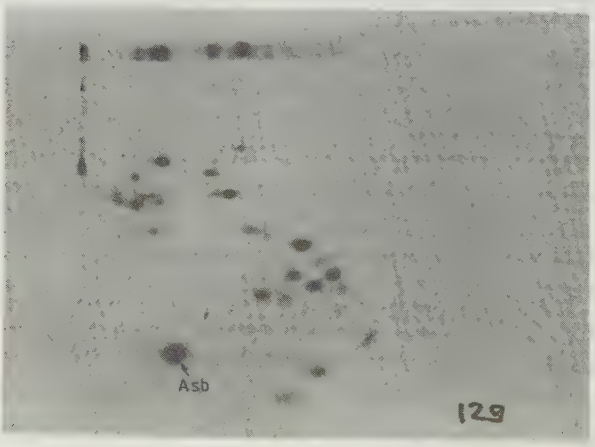


Fig. 33: Asb-protein and 50S-proteins from Th. aquaticus. The Asb-protein clearly co-migrates with the protein AX.

Figure 34 shows the co-migration of Bcb/Bsb in the background of 50S-proteins from B. caldolyticus. The Bcb-protein gives the same result on the co-migration with 50S-proteins as reported for the Bsb-protein from B. stearothermophilus (48). No differences can be seen between the two proteins from the Bacillaceae.

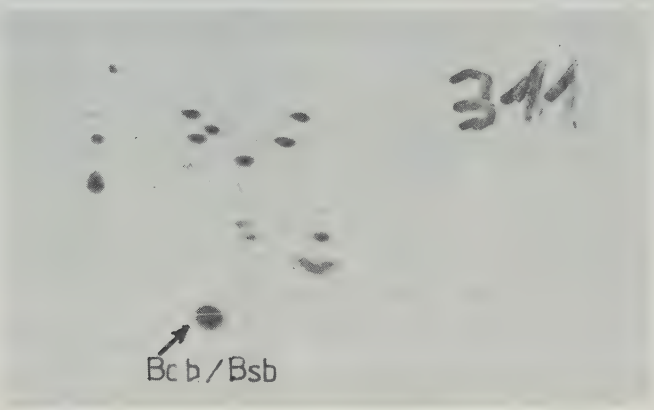


Fig. 34: Bcb/Bsb co-migration in the presence of 50S-proteins from B. caldolyticus.

3.6.2. Determination of the amino acid sequence of the DNA binding proteins

The primary structure of the proteins was characterized by amino acid analysis, N-terminal sequence analysis, enzymatic digestion and peptide mapping followed by sequence analysis of the peptides. In view of the small amount of Asb-protein obtained, only amino acid analysis could be carried out with this protein.

3.6.2.1. Amino acid analysis of the proteins

The analysis was carried out on an acid protein hydrosylate. Therefore, tryptophane was destroyed and the amide-group of asparagine and glutamine were hydrolyzed. Table 1 shows the amino acid composition of Tsb, Asb, Bcb and Csb.

Table 1: Amino acid composition of the isolated DNA binding proteins

	Tsb	Asb	Bcb	Csb
Asx	5.2	4.2	9.4	5.9
Thr	8.5	8.9	5.5	6.1
Ser	3.2	----	3.9	3.4
Glx	8.9	14.2	11.4	14.8
Pro	4.4	2.9	4.9	2.7
Gly	6.1	11.4	7.9	6.1
Ala	18.9	30.1	11.1	10.8
Val	5.1	7.2	6.7	6.7
Met	1.1	0.8	2.1	2.2
Ile	7.1	0.5	4.4	4.4
Leu	6.5	1.3	5.6	6.8
Tyr	0.9	2.4	----	----
Phe	2.4	0.8	4.3	3.9
His	----	----	----	----
Lys	14.3	3.7	8.0	14.2
Arg	2.9	2.9	4.9	5.5
	92.3%	91.3%	90.1%	93.5%

The values are given in mole percentage. Each protein was analysed twice and the average values are presented.

Three of the proteins, namely Tsb, Bcb and Csb, are characterized by a high amount of basic amino acids, such as lysine and arginine which make up for more than 17% of the total sum. A common feature of all proteins is the lack of histidine. The Asb-protein is characterized by an extraordinary high amount of alanine, which represents approximately 30% of the residues. The amount of basic amino acids is low (7%) when compared to the three other proteins. Serine is not present in Asb. The amino acid analysis reveals that this protein is different from other DNA binding proteins such as Bsb (48) and NS1, NS2 (21, 73).

3.6.2.2. N-terminal sequence determination

The N-terminal analysis of intact protein was performed using the Edman degradation technique as modified by Chang et al. (56). The degradation cycles could generally be continued up to the 8th and 9th residue. Table 2 shows the N-terminal amino acid sequences of Tsb, Bcb and Csb:

Table 2: N-terminus of DNA binding proteins from Th. thermophilus, B. caldolyticus and C. pasteurianum

		1	2	3	4	5	6	7	8	9
Tsb:	NH_2 -	Ala -	Ala -	Lys -	Lys -	Thr -	Val -	Thr -	Lys	
Bcb:	NH_2 -	Met -	Asn -	Lys -	Thr -	Glu -	Leu -	Ile -	Asn -	Ala
Csb:	NH_2 -	Met -	Asn -	Lys -	Ala -	Glu -	Leu -	Ile -	Thr -	Ser

The sequence homology between Bcb and Csb is obvious. The first 3 residues are identical, and conservative exchanges occur in residues 4, 8 and 9. On the other hand, the N-terminal sequence of Tsb-protein does not agree with the so far studied sequences of the DNA binding protein II from any of the organisms.

3.6.2.3. Enzymic and chemical cleavage of intact proteins

For further determination of the primary structure, Tsb as well as Bcb were digested and the peptides analysed as described (Section 2.12.5.).

3.6.2.3.1. Tryptic peptides of Tsb

The peptides were separated by fingerprinting on thin layer plates. For visualization the peptides were stained with a stock solution of ninhydrin and for sequence analysis with fluorescamine (Section 2.12.5.).

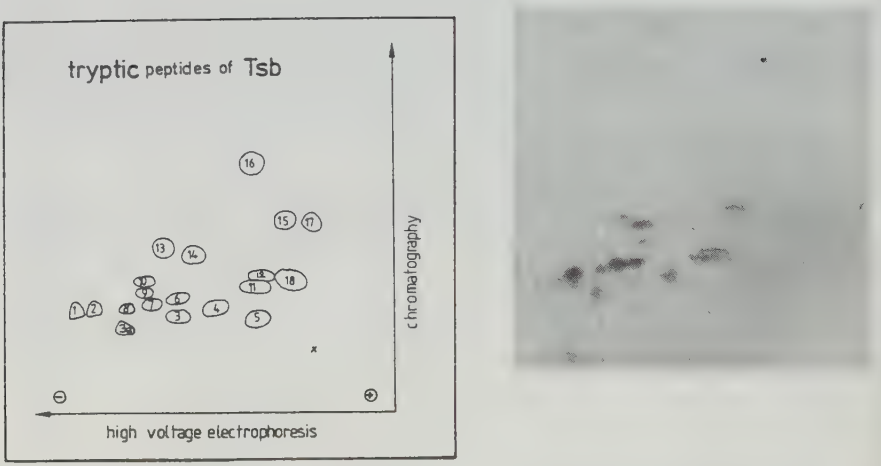


Fig. 35: The tryptic fingerprint of Tsb-protein, 2 mg of Tsb were digested with trysine and employed for high voltage electrophoresis and thin layer chromatography. The peptides were stained with 0.1% ninhydrin. On the left hand side, the schematic chromatogram.

The protein was cleaved into small basic peptides as can be seen in Figure 35. For sequence analysis the peptides were scraped and extracted with 50% acetic acid. The amino acid composition as well as the N-terminal amino acid of the peptides are shown in Table 3.

Table 3: Amino acid composition of tryptic peptides of Tsb-protein

Amino acid	Peptide position																	
	T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8	T_9	T_{10}	T_{11}	T_{12}	T_{13}	T_{14}	T_{15}	T_{16}	T_{17}	T_{18}
N-terminus	Arg			Glu	Asp	Glu	Thr	Lys	Ala	Thr	Asp	Val	Ala	Ile	Ala	Ala	Val	Val
Asx			0.8		1.1	1.0					2.2	1.1			2.0	1.2	1.0	1.0
Thr							0.9	0.9	0.5	1.8		0.9		0.6	1.2		2.8	0.9
Ser											1.1	1.1						1.2
Glx				0.9		1.1					1.0	2.1		0.6	2.3		2.5	2.2
Pro							0.5							1.7				
Gly						1.4	0.9		0.7			1.2		0.6	1.2		2.6	1.2
Ala							1.0		1.0			1.7	1.0	1.0	2.8	3.4	1.4	2.2
Val		1.1.	0.7				1.0	0.7		0.9	1.0	0.9			1.6	1.0	2.7	0.8
Met																1.1		
Ile						0.6							1.4					
Leu						0.9						0.8	1.4		1.8	2.1	1.9	0.9
Tyr						1.7								0.7				
Phe														0.6			2.4	
Lys	0.6	2.2	1.1	1.0	0.8	2.2	1.4	1.8	0.6	0.9	1.2	0.9	1.2	1.1	0.9	1.1		1.0
Arg	1.1								0.8								1.1	

Amino acid sequence of tryptic peptides: A few peptides were difficult to sequence since they possess hydrophobic amino acids, which led to increasing losses during Edman degradation. Only those peptides that could be successfully sequenced, are listed (→→: sequenced by manual Edman degradation):

T_1 Arg - Lys
→

T_4 Glu - Lys
→

T_5 Asp - Lys
→

T_7 Thr - Gly - Val - x - Pro - Gly - Thr, Ala, Lys
→ → → → → →

T_8 Lys - Thr - Val
→ → →

T_9 Ala, Thr, Gly, Lys, Arg
→

T_{10} Thr - Val - Thr - Lys
→ → →

T_{11} Asp - Val - Glu - Asp, Ser, Lys
→ → → →

T_{12} Val - Glu - Glu - Leu - Ala - Leu - Asp, Lys, Ser, Gly, Thr
→ → → → → → →

T_{13} Ala - Leu, Ile, Lys
→ →

T_{14} Ile - Pro - Ala - Thr - Gln - Tyr - Pro - Ala - Phe - Gly, Lys
→ → → → → → → → → →

T_{15} Ala - Asp - Gly - Leu - Val - Asp - Gln - Val - Ala - Gln - Ala - Thr - Gly - Leu, Lys
→ → → → → → → → → → → → → →

T_{16} Ala - Met - Val - Asp - Ala - Leu - Leu, Lys
→ → → → → → →

T_{17} Val - Gln - Leu - x - Val - Gly, Asx, Thr, Gly, Ala, Phe, Arg
→ → → → →

T_{18} Val - Glu - Glu - Ala - Leu - Ala - Asn - Gly - Ser, Thr, Lys
→ → → → → → → → →

3.6.2.3.2. Peptic peptides of Tsb

Tsb-protein was digested with pepsin and the peptides were separated as described for the tryptic digest.

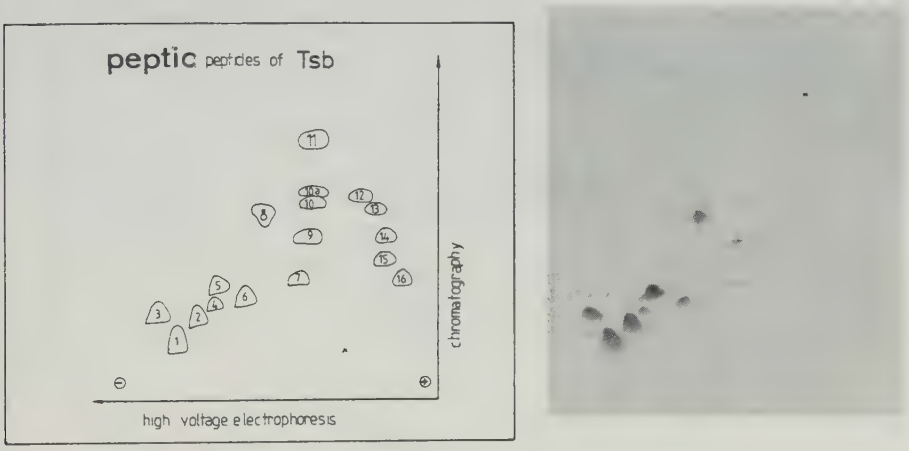


Fig. 36: The Tsb-peptides were separated by high voltage electrophoresis in the first dimension and by chromatography in the second. The peptides were stained with 0.1% ninhydrine and numbered as in the schematic drawing.

Amino acid sequence of peptic peptides: For sequence analysis the peptides were extracted as described in Section 2.12.5. Table 4 shows the amino acid analysis of the peptides.

Table 4: Amino acid composition of peptic peptides

Amino acid	Peptide position															
	P_1	P_2	P_3	P_4	P_5	P_6	P_7	P_8	P_9	P_{10}	P_{10a}	P_{11}	P_{12}	P_{13}	P_{14}	P_{15}	P_{16}
N-terminus	Glu	Glu	Pro	Val	Ala	Val	Ala	Ala	Leu	Thr		Thr		Val	Val	Val	Val
Asx		1.1	1.1	1.1	1.2	1.1		1.1				1.0		0.8	0.7	0.8	1.0
Thr	0.5	1.3		0.6	2.2			3.1	2.7	2.4	2.4	1.8		0.7			
Ser			0.8		1.1										0.6	0.8	0.4
Glx	0.9	2.4			0.8	0.6	1.2	1.2	1.0					1.3	1.4	0.9	1.1
Pro		1.2	1.0														
Gly		2.0	1.4	1.2		0.9		1.2	0.5	2.7	2.7	2.3	0.8	1.4	1.4	1.1	1.9
Ala	2.5	1.5	2.3	2.5	3.5	1.9	0.8	2.5	1.3		1.1	1.0	1.3	0.6	0.6		
Val	0.9	1.1	0.9	1.0	1.0	1.8	0.5	1.1						1.3	1.4		
Met						1.0							0.9				
Ile	0.5	0.9															
Leu	0.5	0.9	0.8					2.3	0.6					1.1			
Tyr		0.6															
Phe			0.9						2.2			1.0					
Lys	2.5	2.6	3.5	2.0	3.2	1.6	1.2	1.0	0.6								
Arg	1.0	1.4															

Many of the peptides were not sequenced since they were lost by manual Edman degradation. For some peptides only the N-terminal residue could be determined. Table 5 shows the peptides which were analysed.

Table 5:

P_1	Glu - Val, Thr, Ile, Leu, $(Lys)_2$, Arg
P_2	Glu - Val - Thr - Lys, Asx, Glx, Pro, $(Gly)_2$, Ala, Ile, Leu, Thr, $(Lys)_2$
P_3	Pro - Ala - Leu - Lys - Val - Gly - x - Ala, Asx, Phe, Ser, $(Lys)_2$
P_4	Val - Ala - x - Ala, Asx, Thr, Ser, Glx, Gly, $(Lys)_2$
P_5	Ala - Ala - Lys - Lys - Thr - Val - Thr - Lys, Ala, Ser, Asx, Glx
P_6	Val - Asp - Gln - Val, Ala, Fly, Leu, Met, Lys
P_7	Ala - Lys - x - Val, Glx, Gly, Ala
P_8	Ala - Leu - Leu - Ala - x - Gly, Asx, Glx, $(Thr)_3$, Val, Lys
P_9	Leu - Gly - x - Val, Asx, $(Thr)_3$, Ala, Glx
P_{10}	Thr, Gly, Ala, Gly, Thr
P_{11}	Thr - Gly, Asx, Thr, Gly, Ala, Phe
P_{13}	Val - Asp - x - Val, Thr, Ser, Glx, Gly, Ala, Leu
P_{14}	Val - Asp - Gln - Val, Ser, Gly, Ala
P_{15}	Val - Asp - Gln, Ser, Gly
P_{16}	Val - Asp, Gly, Ser, Glx

3.6.2.3.3. Cyanogen bromide cleavage of Tsb

Tsb-protein was treated with a 20 molar excess of BrCN (Section 2.12.4.3.). After 20 hr. the mixture was lyophilized and checked for composition by SDS-slab-gel electrophoresis.

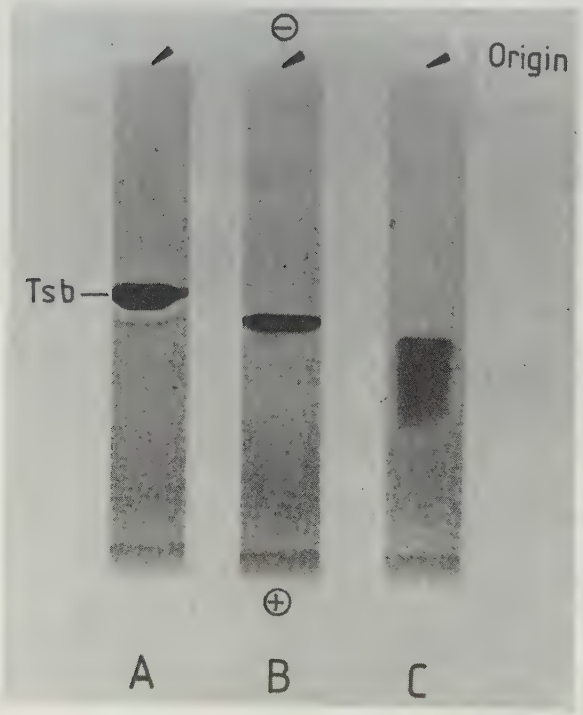


Fig. 37: SDS-slab-gel electrophoresis (20% acrylamide) of BrCN cleaved Tsb. The mixture was separated on a Sephadex-G-50-column in 10% acetic acid.

(A) intact Tsb-protein;
(B) C-terminal fragment;
(C) N-terminal fragment.

Most of the protein was cut into two fragments of different molecular weight (3000 and 6500 dalton), which could be easily separated by gel filtration. Fractions containing pure peptides were pooled and a small aliquot used for amino acid analysis. The remainder was used for manual solid-phase sequencing.

Table 6: Amino acid composition of the small fragment (N-terminus)

amino acid	mol %	amino acid	mol %
Asx	2.5	Met (homoserine)	1.0
Thr	3.0	Ile	---
Glx	2.5	Leu	2.7
Pro	---	Tyr	---
Gly	1.1	Phe	---
Ala	4.9	Lys	4.9
Val	2.2	Arg	---

homoserine was eluted immediately before glutamic acid.

Sequence analysis of the N-terminal homoserine fragement: the carboxyl-group of the homoserine fragment was coupled to aminopropylglass and sequenced by manual Edman degradation. The complete sequence of the fragment could be obtained.

NH_2 - Ala - Ala - Lys - Lys - Thr - Val - Thr - Lys - Ala - Asp - Gly - Leu - Val - Asp - Gln - Val - Ala - Gln - Ala - Thr - Gly - Leu - Leu - Lys - Ala - COOH

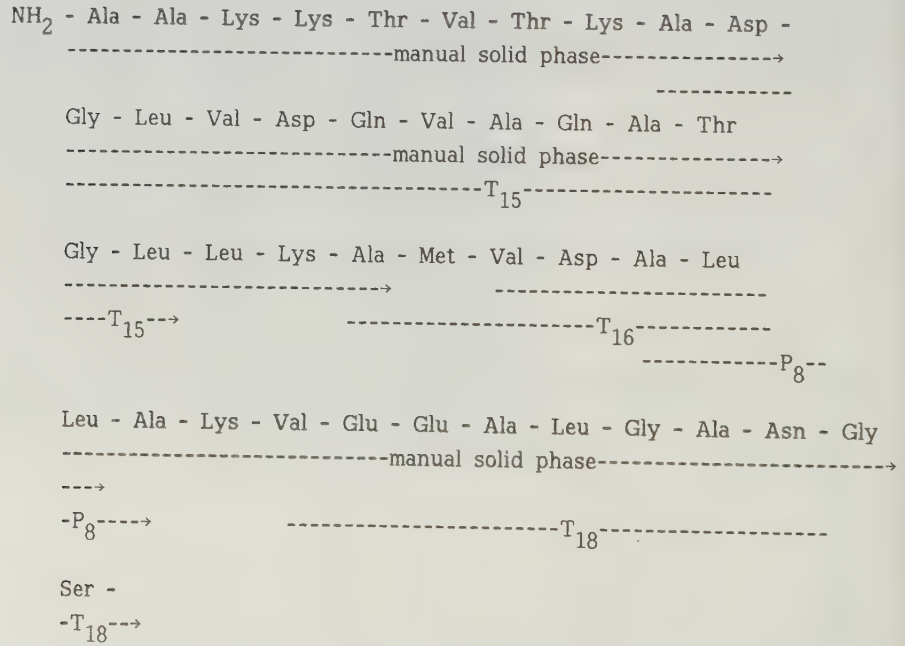
Table 7: Amino acid of the C-terminal fragment

amino acid	mol %	amino acid	mol %
Asx	2.75	Met	---
Thr	5.5	Ile	1.6
Ser	2.2	Leu	4.6
Glx	6.4	Tyr	0.9
Pro	4.0	Phe	2.4
Gly	5.0	Lys	9.4
Ala	6.7	Arg	2.8
Val	2.9		

For the determination of the amino acid sequence of the large peptide (Fig. 37, B), the C-terminal carboxyl-group was activated and attached to the glass support. Only a small part of the peptide was sequenced with this procedure. After the 15th degradation step, no amino acid could be detected:

NH_2 - Val - Asp - Ala - Leu - Leu - Ala - Lys - Val - Glu - Glu - Ala - Gly - Ala - Asn - Gly

The complete N-terminal sequence of protein-Tsb up to the 42nd residue is given in Table 8.



3.6.2.3.4. Tryptic peptides of Bcb

The digestion of the intact protein and the separation of peptides were performed as described above. Figure 38 illustrates the chromatogram of the tryptic peptides from Bcb.

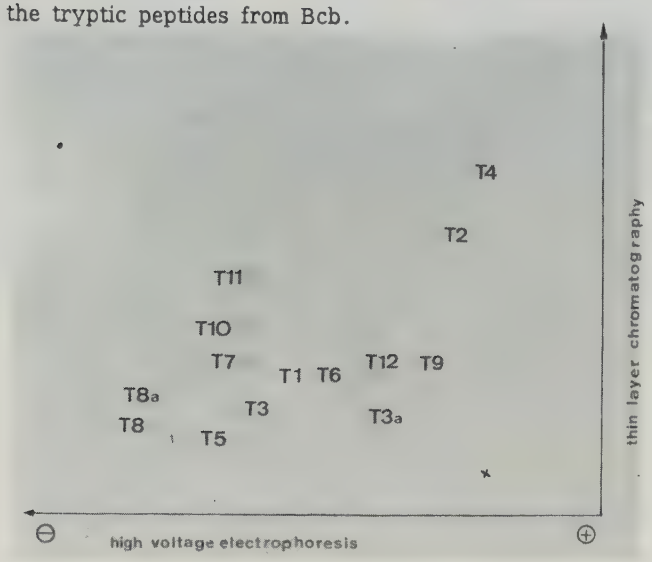


Fig. 38: Tryptic peptides of Bcb were separated by high voltage electrophoresis and thin layer chromatography

Table 8 shows the amino acid analysis of the peptides, compared with the corresponding peptides of Bsb. The peptides T_4, T_5, T_{10} and T_{11} exhibit several differences to the corresponding peptides of Bsb. Peptide T_4 from Bcb has no phenylalanine, T_5 has neither isoleucine nor leucine, peptide T_{10} has no glycine and peptide T_{11} has no leucine. Since most of the protein was used for crystallization, no further investigation on the sequence was done.

3.6.3. Circular dichroism spectroscopy: the effect of increasing temperature and of urea on the structure of the DNA binding proteins.

CD-experiments were performed with a Cary 60 spectrometer (Section 2.14). Structural changes were monitored using the reading at 220 nm which is mainly contributed by α-helices. For temperature denaturation, the cell compartment was heated in steps of 5 degrees. The Tsb-protein shows no loss of the α-helical amount up to 30°C. A gradual loss of the α-helical amount can be seen at temperatures above 30°C, as it was re-

Table 8: Tryptic peptides of Bsb and Bcb (Bsb data from Kimura and Wilson, (23)

Amino acid	Peptide position														
	T_1	T_2	T_3	T_{3a}	T_4	T_5	T_{5a}	T_6	T_7	T_8	T_{8a}	T_9	T_{10}	T_{11}	T_{12}
Asx	1.04 1.00	1.22 1.00	1.11 1.00	1.00 1.00	1.83 2.00	2.43 2.00	0.77 0.90					0.77 1.00			0.96 Bsb/ 1.00 Bcb
Thr		1.82 1.32	1.40	1.18 0.87	1.00 0.90							0.85 1.00			
Ser		1.77 1.40			0.87 0.99							0.82 1.19			
Glx		2.35 1.80			1.05 1.15	1.71 2.26		1.18 1.00				3.84 4.09			
Pro												1.79 2.00	1.78 2.20		
Gly		1.21 1.06				3.39 1.14	1.00			1.29 1.24	1.07	1.00 1.50	1.40		
Ala		2.00 1.76	1.07 1.00	0.95	2.63 2.90				1.88 1.95			1.02 1.25	1.25 1.00	0.92 1.00	1.04 1.02
Val		1.06 0.94			2.02 1.28	1.61 2.24							0.93 0.99		1.09 1.03

Table 8 continued: Tryptic peptides of Bsb and Bcb (Bsb data from Kimura and Wilson, (23)

Amino acid	Peptide position															
	T_1	T_2	T_3	T_{3a}	T_4	T_5	T_{5a}	T_6	T_7	T_8	T_{8a}	T_9	T_{10}	T_{11}	T_{12}	
Met	0.75 1.55											0.89 1.00				Bsb/ Bcb
Ile		1.02 0.69			9.99 0.83	0.99						0.89 0.89				
Leu		1.97 0.92			1.04 0.79	0.98								0.95		
Tyr																
Phe					1.01	2.35 2.00							1.24 1.34			
His																
Lys	1.00 0.82 0.98 0.74	1.46 1.42				1.65 1.65	1.90 0.87			0.77 0.79		0.75 0.96	2.34 1.82	1.24 1.05	1.0 0.72	
Arg					0.96 0.97	1.12 1.11		0.83 0.81	1.00 1.00	1.00 1.00	1.00					

ported for the DNA binding protein from E. coli and B. stearothermophilus (48). At 77°C, the main part of the secondary structure starts to "melt", as indicated by the rapid decrease of the ellipticity at 220 nm. The inflexion point of the curve lies at approximately 85°C and the shape of the curve indicates that the melting is highly cooperative. The transition temperature is in good agreement with the maximal growth temperature of Th. thermophilus (85°C). The Bcb reveals no loss of the α-helical structure up to 50°C. Above 50°C, a slow decrease in the amount of α-helix can be seen. The helix-coil transition is characterized by the inflexion point of the melting profile at 67°C.

Csb-protein is stable up to 38°C. Further temperature increase causes a gradually diminution of the α-helix amount. The helix-coil transition is not cooperative, as is the case for Bcb and Tsb. The transition temperature lies at 53°C.

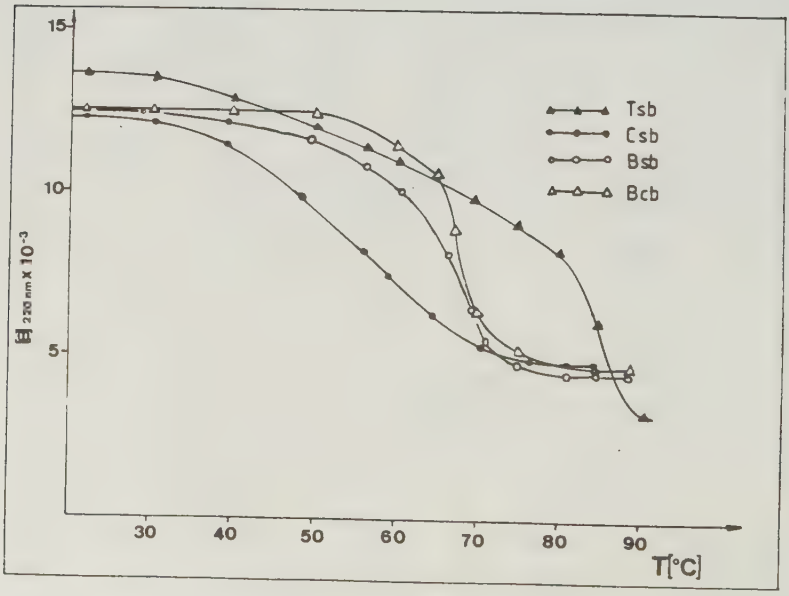


Fig. 39: Thermal denaturation of Tsb-, Csb-, Bsb- and Bcb-protein. The temperature is plotted versus the ellipticity at 220 nm.

When the protein samples are placed in the original environment the ellipticity returns to that amount obtained before heating.

The denaturation was also performed by the addition of urea buffer (Section 2.14.) in the concentration range of 0.25 - 5 M; respectively. As can be seen in Figure 39a, the Tsb-protein is stable up to 2.5 M urea.

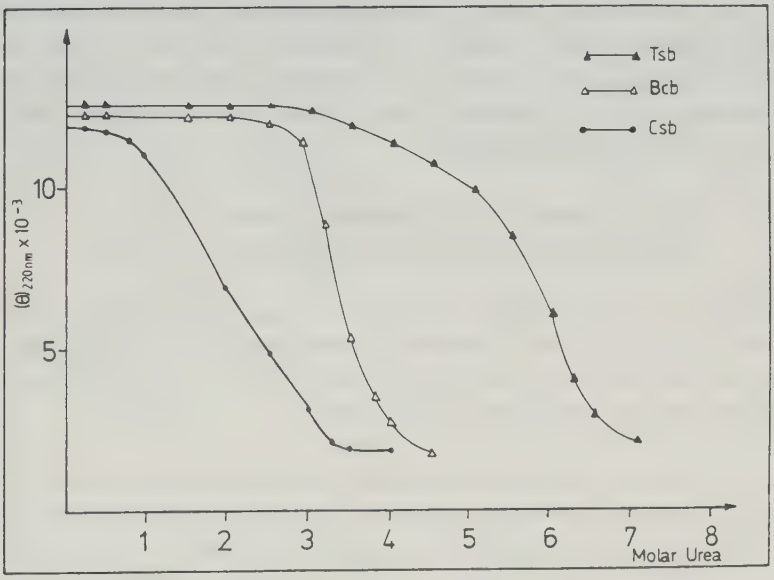


Fig. 39a: Denaturation of Tsb, Bcb and Csb by urea.

From there, the denaturation proceeds gradually at increasing urea concentrations. At 5 M urea and higher, the amount of α-helix decreases sharply. The "melting" point is reached at 5.75 M urea.

The Bcb-protein is stable up to 2.5 M urea. Further increase of the urea concentration is reflected by a slight diminution of the α-helical amount. The helix-coil transition lies at 3.5 M urea.

The Csb-protein is stable up to a urea concentration of 0.75 M. Further addition of urea causes a decrease of the ellipticity at 220 nm. At a urea concentration of 2 M, the protein undergoes denaturation.

The original spectrum is obtained after dialysis of the denatured protein samples against buffer without urea, indicating complete renaturation (Section 2.14.). The high transition temperatures of Tsb (85°C) and Bcb (67°C) are supported by the high urea concentration required for the denaturation. The binding proteins from thermophilic organisms are supposed to have a highly folded internal structure which is resistant to high temperatures as well as to urea. Similar behaviour have been reported for ribosomal as well as for the Bsb-protein from B. stearothermophilus (48, 72). The Csb-protein from C. pasteurianum should possess a less stable structure which is more sensitive to temperature increase as well as to chaotropic agents, such as urea. The melting temperature of the individual proteins is in good agreement with the growth temperature of the bacteria from which they are isolated.

3.6.4. PMR-spectroscopy of the individual proteins and protein/oligodesoxynucleotide complexes

The spectra were recorded at 270 MHZ at protein concentrations of 0.5 - 1.5 mM. Because of the requirement of large amounts of material only the Tsb- and the Bcb-protein were investigated. Figure 40 shows the PMR spectrum of native Tsb-protein in D_2O-buffer.

Resonance peaks of the non-exchangeable amide protons are recorded between 10 - 8 ppm. The ring protons of phenylalanine and tyrosine resonance between 7.3 and 6.8 ppm. The complexity of these resonances indicates perturbance due to folding. The high-field part of the spectrum (2 - 0 ppm) shows perturbed and broadened peaks of methyl protons of hydrophobic amino acids which are influenced by the ring current from aromatic side chains. The methyl protons of alanine, threonine and glutamic acid show broadened peaks between 1 - 2 ppm. A well-separated, large and rather broad peak at 3 ppm, which is attributed to the ε-methylene-protons of lysine, is poorly resolved from a shoulder-like peak at 3.2 ppm. This resonance peak is from γ-methylene-protons of arginine. The part of the molecule which is immobilized by intra- or intermolecular binding sites is characterized by the methyl resonances of hydrophobic amino acids. The presence of a tertiary fold is indicated by the ring current shifted methyl protons of the hydrophobic amino acids and the perturbed and broadened peaks of aromatic amino acids.

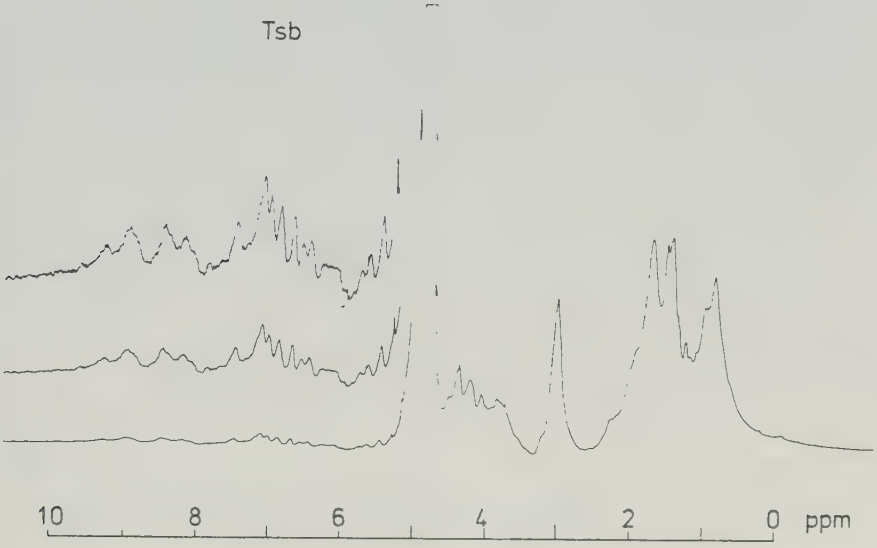


Fig. 40: Native Tsb-protein. The low-field region was recorded in 3 different gain settings.

The broad lysine peak at 3 ppm does not occur usually, for instance not in the Bsb-protein (48). The broadening of this peak could be due to the different environment of the lysine residues in the molecule. Strong evidence for the existence of a highly folded structure is the presence of many, poorly resolved amide proton resonances in the 8 - 10 ppm region. This phenomenon is rather characteristic for thermophilic proteins, e.g. ribosomal proteins from B. stearothermophilus (72).

Figure 41 shows the PMR spectrum of the urea-denatured Tsb-protein.

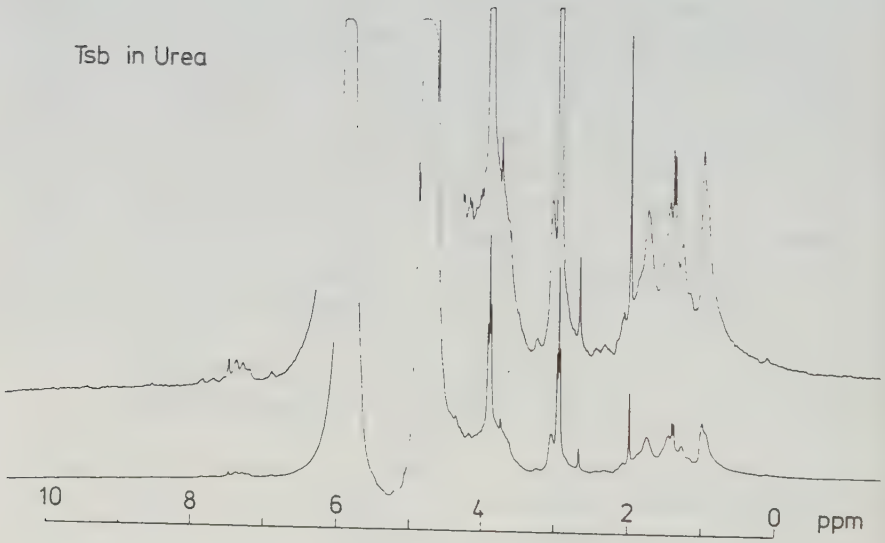


Fig. 41: Tsb-protein in 8 M urea. The low-field and high-field region is recorded in two different gain settings. The low-field part, 8 - 10 ppm, shows the loss of amide protons which have been exchanged with the buffer.

The spectrum shows the sharp, well-resolved peaks of the random-coil conformation with none of the perturbations' characteristic for a folded structure. The high-field part shows clear and sharp peaks of methyl protons of the hydrophobic amino acids. The ε- as well as δ-methylene protons of lysine and arginine give well-resolved signals at 3.0 and 3.2 ppm, respectively. The loss of the amide proton resonances and the clear, well-resolved signals are characteristic for a random-coil conformation. However, an internal structure of parts of the molecule cannot be excluded since there are still sequence effects in the low-field region.

Figure 42 shows the spectrum of Tsb in the presence of adenine/thymidine desoxynucleotides in a molar protein:nucleotide ratio of 1:2. The low-field

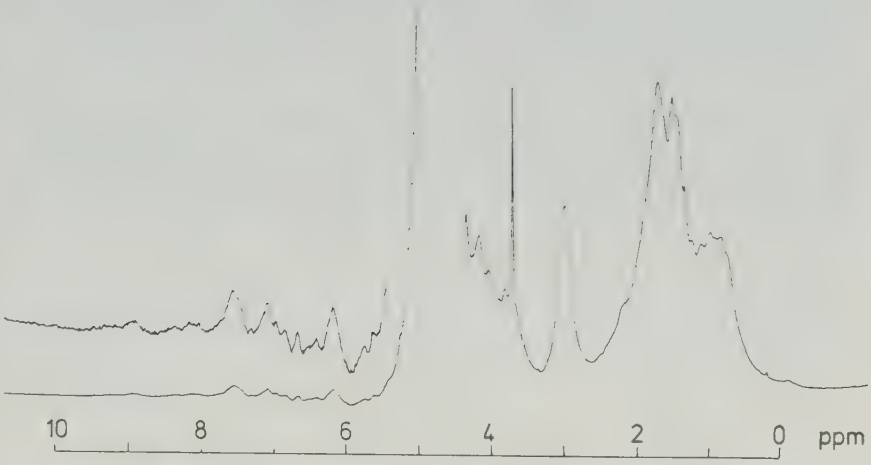


Fig. 42: Tsb-protein in the presence of dA_8/T_8. The low-field part, 8 - 10 ppm, is recorded in two different gain settings.

part of the spectrum is characterized by a decrease of the amide proton signal from the poly peptide backbone. A substantial part of the original peptide bond signal is lost. The aromatic resonances of phenylalanine and tyrosine are broadened and shifted downfield by 0.1 ppm.

The high-field part is characterized by broad peaks of the hydrophobic amino acids. Ring current shifts of the methyl protons show no change in the environment of aromatic and hydrophobic amino acids. The lysine signal appears in the original size, but slightly broadened, at 3 ppm.

Those results provide support for the following conclusions: (1) A partial conformation change, caused by the oligonucleotide binding cannot be excluded since there is a decrease of the amide proton signal at 8 - 10 ppm. (2) Hydrophobic amino acids, such as isoleucine, leucine and valine are effected either by conformational change or by nucleotide binding, which can be seen by the broad resonances. (3) The distance between aromatic rings and hydrophobic amino acids is probably uneffected. This is re-

flected in a lack of alteration in ring current shifts. (4) The signal of the lysine residues at 3 ppm is slightly broadened. This might indicate small conformational changes in the environment of the lysine residues within the molecular structure.

The Bcb-protein spectra are completely different from the Tsb-protein. The patterns of the native, of the urea-denatured and of the oligonucleotide-bound protein are identical with the corresponding spectra from Bsb (48).

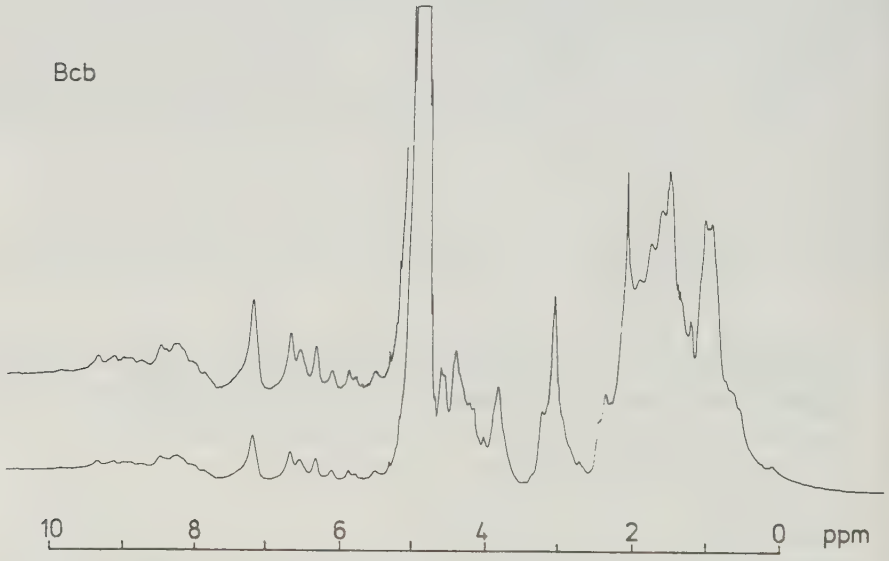


Fig. 43: Native Bcb-protein; the low-field is recorded in two different gain settings.

Figure 43 shows the native spectrum of Bcb. Poorly resolved resonance peaks between 8 and 10 ppm arise from non-exchangeable amide protons of the polypeptide backbone. Broad and perturbed resonances of phenylalanine can be seen at 7.3 and 6.8 ppm. A large number of poorly resolved ring current shifted methyl resonances at 0 - 1 ppm are present. The resonance peaks of the ε-methylene protons of lysine are well-resolved at 3 ppm. The δ-methylene protons of arginine give a small signal at 3.2 ppm.

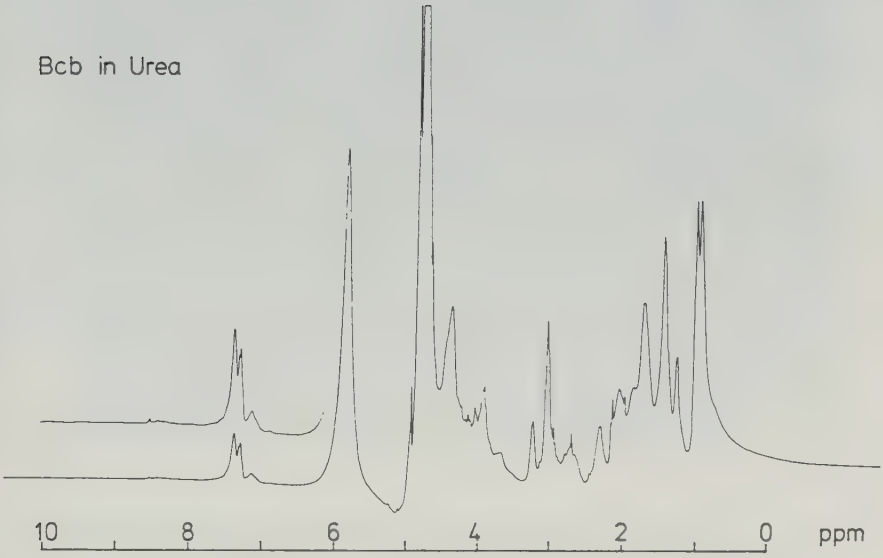


Fig. 44: Bcb-protein in the presence of 8 M urea. The low-field is recorded in two different gain settings and characterized by the loss of amide proton resonances.

Figure 44 shows the urea denatured spectrum of Bcb, which has the same features as the corresponding Bsb-spectrum (48). Perturbed and broad peaks are lost, as it would be expected for a denatured protein. The aromatic protons appear at 7.3 ppm. The high-field part is characterized by sharp and well-resolved peaks of the resonance from methylene and methyl protons.

Figure 45 shows the spectrum of Bcb in the presence of adenine/thymidine desoxynucleotides. The molar ratios are as described for Tsb. The spectrum indicates that there is no essential difference in the spectrum after binding of the nucleotides.

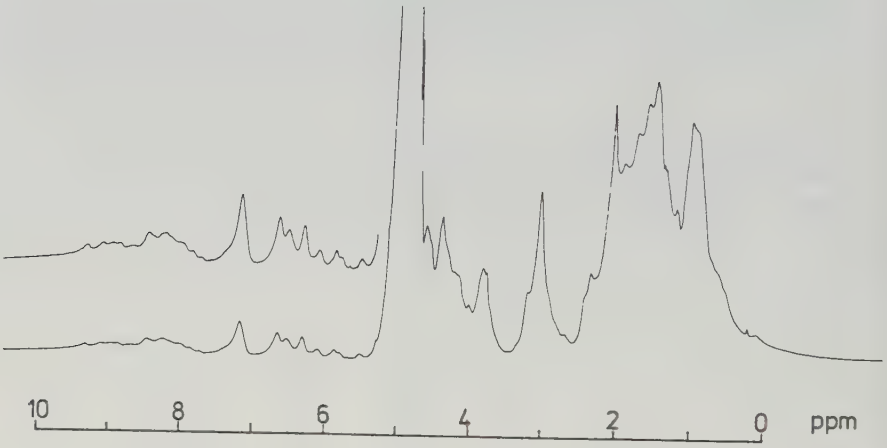


Fig. 45: Bcb in the presence of dA_8/T_8. The low-field region is recorded in two different gain settings.

Between the spectra of proteins with and without binding of nucleotides only one difference can be seen at the slight shift high-field arginine resonance at 3.2 ppm, as well as a small decrease of the methylene proton resonances of hydrophobic amino acids. Similar changes in the PMR spectrum have been reported for the Bsb-protein (48).

3.6.5. Crystallization of proteins

Proteins were crystallized at concentrations of 4 and 30 mg/ml using the vapour diffusion technique (Section 2.16.).

3.6.5.1. Tsb crystallization

The protein was crystallized using three different precipitant solutions (Section 2.16.). Crystals (Fig. 46) were obtained at a protein concentration of 4 mg/ml from 3.0 M $(NH_4)_2SO_4$ (solution 1). The crystals were too small (0.05 x 0.05 x 0.1 mm) for X-ray analysis.

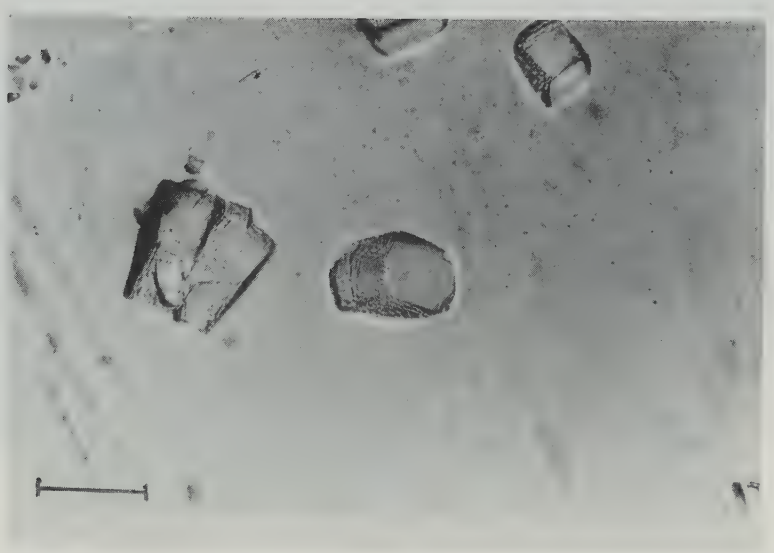


Fig. 46: Tsb crystals, obtained by vapour diffusion against 3.2 M $(NH_4)_2SO_4$ at 20°C. The protein concentration per droplet was 2 mg/ml. Bar length: 0.1 mm.

Crystals of a different shape (Fig. 47) were obtained at the same protein concentration from 3.2 M $(NH_4)_2SO_4$ (solution 2). Small crystals (0.05 x 0.05 x 0.1 mm) first appeared after 5 months at 18°C. The growth of the crystals then stopped after an additional period of 1 month.

Larger crystals were obtained at a protein concentration of 30 mg/ml under the same conditions (solution 2). Crystals suitable for X-ray analysis were grown at an ammonium sulfate concentration in the range of 3.0 - 3.4 M. The dimensions of the crystals were 0.1 x 0.1 x 0.3 mm. The shape and the diffraction pattern of one of the 6 crystals obtained can be seen in Figure 48.

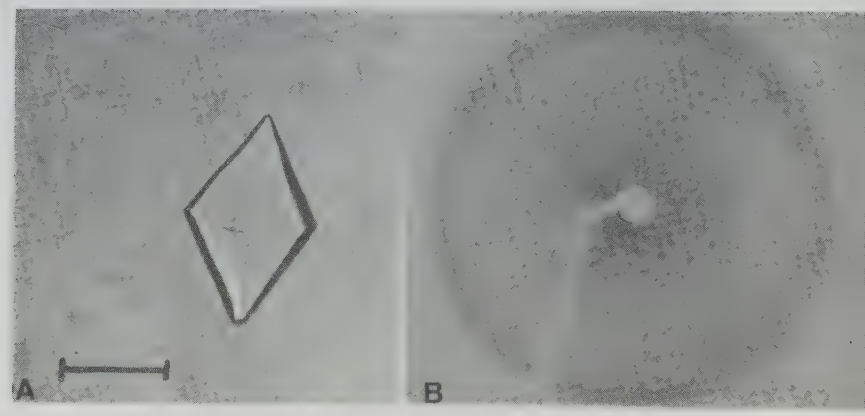


Fig. 47: (A) Tsb crystal, obtained after 6 months at 18°C by vapour diffusion against 3.2 M $(NH_4)_2SO_4$. Protein concentration per droplet was 2 mg/ml. (B) diffraction pattern of the crystal. Bar length: 0.1 mm.

Table 9: The crystallographic data of Tsb-protein, as determined from procession photographs.

Space group			P 622	
lattice constants	a	=	64.1	(γ = 120°)
	b	=	64.1	
	c	=	116.2	
Volume of the cell unit		=	412.950 Å 3	
Resolution about 3 Å				

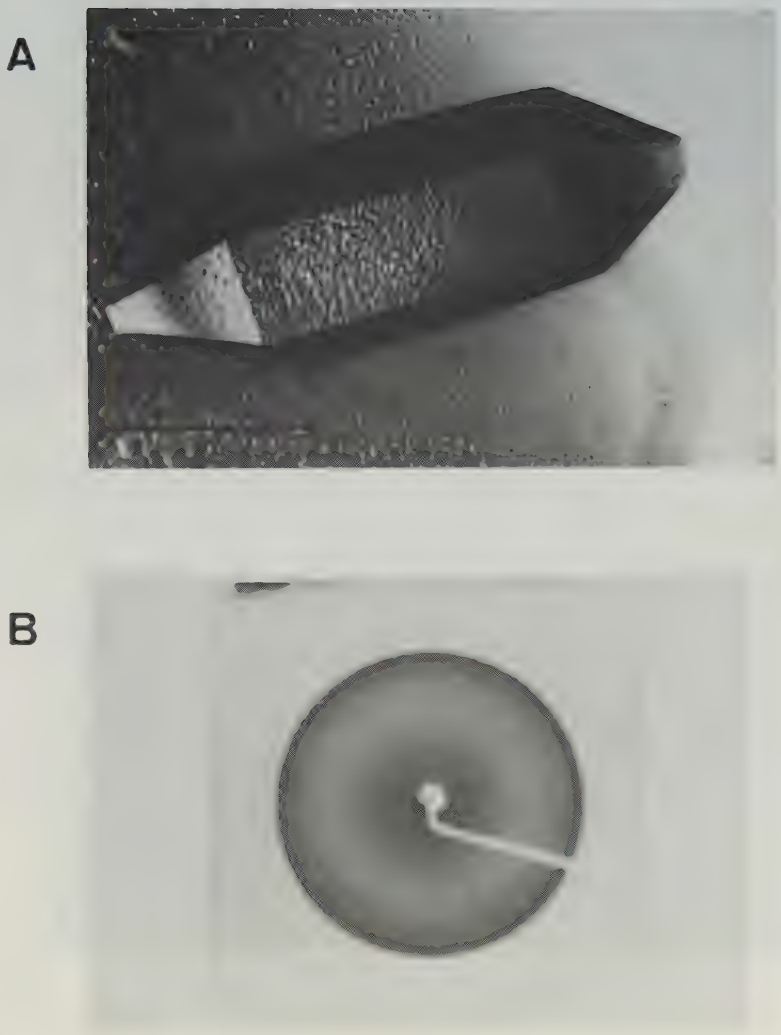


Fig 48: (A) Tsb crystal, obtained after 5 months by vapour diffusion against 3.2 M $(NH_4)_2SO_4$. Protein concentration per droplet: 15 mg/ml. (B) diffraction pattern of the crystal; distance between crystal and film: 100 mm. Bar length: 0.1 mm.

3.6.5.2. Bcb crystallization

Protein droplets (30 mg/ml) were equilibrated using vapour diffusion with MPD-buffer (solution 3, section 2.16.). Two morphological crystal forms were obtained at 20°C after 2 months. Figure 49 shows the crystals grown in the presence of 30%, as well as 36%, MPD. The protein crystallizes in the shape of long needles as well as plates. Both crystal forms are stable within the temperature range of 10°C - 20°C over a time period of more than 4 months. The same observation has been reported for the Bsb-protein from B. stearothermophilus (48, 75).

A

B

Fig. 49: Bcb crystals grown over a time period of 2 months from MPD; (A) plates are obtained by vapour diffusion against 30% MPD; (B) the protein crystallizes in a needle-like form at a MPD concentration of 36%. Protein concentration per droplet: 15 mg/ml. Barlength: 0.1 mm.

3.6.5.3. Csb crystallization

Vapour diffusion experiments with $(NH_4)_2SO_4$ and MPD (solutions 1 and 3) were carried out at protein concentrations of 4 mg/ml. Crystallization conditions were varied between 2.0 - 3.2 M $(NH_4)_2SO_4$ and 18 - 38% MPD. No crystalline material was observed in the experiments. In view of the small amount of protein available, further crystallization efforts had to be abandoned.

4. Discussion

4.1. Purification of ribosomes and the DNA binding proteins

Several methods have been developed for the isolation of ribosomes. Most all of these require high salt to remove non-specifically bound proteins from the surface of the ribosomes. It can not be ruled out with certainty that under these conditons either some loss of ribosomal proteins occurs and non-ribosmal proteins remain attached to the ribosomes. The purification procedure described in this paper relies on gel filtration of cytoplasmic extracts in the presence of high salt (28, 48). With this procedure it was possible to remove most of the cytoplasmic proteins from the ribosome. The DNA binding proteins, initially attached to the ribosomes but removed by high salt wash, eluted from the column in the post-ribosomal fractions where they were identified by their migration on the SDS-slab-gel. The purification of these proteins was generally carried out by ion exchange chromatography followed by gel filtration. Obviously the proteins show different binding affinities on the ion exchanger. The Tsb-protein from Thermus thermophilus, grown at 80°C, was purified on a CM-Sepadex-C-25- as well as a Heparin-Sepharose-Cl-6B-column. No differences between the proteins isolated from the ion exchanger or from the affinity column were seen. The corresponding protein was not detected in the post-ribosomal fractions from cells grown at 55°C instead of 50°C. At the elution volume of Tsb, two proteins appeared with a molecular weight of approximately 9000 (calculated from the migration on the SDS-slab-gel electrophoresis, Tsb possesses a molecular weight of 10.000). No further investigation was done on those two proteins since they could not be separated even in the presence of 6 M urea and were blocked at the N-terminus.

The extraction of the DNA binding protein from Thermus aquaticus (Asb) was not successful; very little protein was found in the post-ribosomal fractions since apparently it eluted together with ribosomes, as confirmed by 2-D-gel electrophoresis of ribosomal protein extracts. Only a small amount of less than 5 mg eluted at the retention volume of the DNA binding proteins, which was then purified. Since the protein contaminations were not removed by ion exchange and affinity chromatography, only gel filtration in the presence of 20% acetic acid was successful.

In contrast to the DNA binding protein from Bacillus caldolyticus (Bcb), the purification of the corresponding protein from Clostridium pasteurinaum (Csb) was contaminated with cytoplasmic proteins which exhibit similar molecular weights and charge features as the Csb protein. Therefore, the purification was only successful when using a combination of ion exchanger and gel filtration. The Bcb protein was easily purified to homogenity by ion exchange as well as Heparin-Sepharose chromatography as was also used for the isolation of the binding protein from Bacillus stearothermophilus (48). All isolated proteins were soluble, both in water and in salt up to 2 M.

The molecular weight of the DNA binding proteins was determined by SDS-slab-gel electrophoresis. The average value obtained (9.800 dalton) is in good agreement with those determined for the DNA binding protein from Escherichia coli, NS1/2 (21, 73) and from Bacillus stearothermophilus, Bsb, (23).

4.2. Comparison of ribosomal protein composition and stability

The ability of some bacteria to grow at temperatures of 60° - 80°C certainly requires a pronounced degree of thermal stability of their macromolecules. The ribosomes of Bacillus stearothermophilus (76, 77), Thermus aquaticus (62) and of thermophilic fungi (78) are more temperature-resistent than those of Escherichia coli. The thermal stability of the ribosomes has been attributed more to the ribosomal proteins than to their rRNA molecules. This has been demonstrated by the heat stability of hybrid 30S subunits of 16 rRNA and proteins from Escherichia coli and Bacillus stearothermophilus (79).

Firstly, the stability of ribosomes of Thermus thermophilus grown at 80°C, as well as at 55°C, and their protein composition were studied. Then we performed comparative studies on the total ribosomal protein extract of the 4 different organisms. A comparison of the patterns from 2-D-gel electrophoresis is useful for a phylogenetic study of closely related bacteria, as has been reported for the Enterobacteriaceae (16, 17). Whereas a comparison of the 5S rRNA sequences can be misleading for this purpose, since this molecule has been conserved over a wide range of organisms. For example, there are no differences in the 5S rRNA sequences of Escherichia

coli, strain CA2 and C1; and only two nucleotide substitutions in the 120 nucleotide length can be seen between Escherichia coli, strain C 1, and Salmonella typhimurium (5, 81).

The thermal denaturation studies on 70S ribosomes from Thermus thermophilus, grown at 80° and 55°C, reveal differences in the melting profiles. Ribosomes from cells grown at 80°C are stable up to the optimal growth temperature (80 - 81°C) and have a transition temperature of 89°C, whereas the ribosomes from cells grown at 55°C are only stable up to 50°C. The melting from the latter was not as cooperative and it occurred at 80°C.

For further investigation the ribosomal proteins from both batches grow at 80°C or 55°C were extracted. The co-migration pattern of the protein extract from 70S ribosomes is shown in Figure 50.

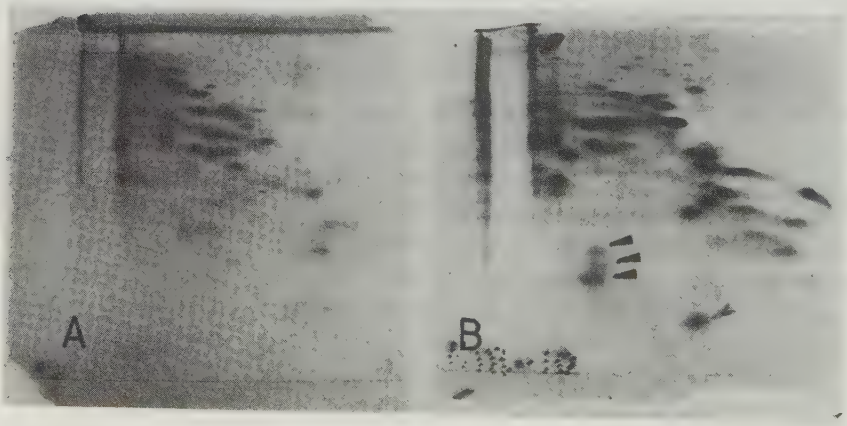


Fig. 50: Proteins from 70S ribosomes grown at 80°C (A) and co-migration of ribosomal proteins from cells grown at 80° and 55°C (B). Arrow marked spots are additional proteins on ribosomes from cells grown at 55°C.

As can be seen, there are 4 additional proteins, at least on the ribosomes from cells grown at 55°C. This could be due to a heterogeous population of ribosomes.

For a comparison of the evolutionary relationship of the 4 different organisms by 2-D-gel electrophoresis of the ribosomal protein extract it was necessary to obtain ribosomes without cytoplasmic contaminations. As can be seen in Figures 11 - 15 (Section 3.3.2.), the patterns are almost free of high molecular weight proteins. On the other hand, the appearance of the Asb-protein from Thermus aquaticus on the 30S and 50S subunit (AX in Fig. 12), indicates that not all low molecular weight proteins might be removed even in the presence of high salt.

The 30S subunits of the Gram-negative bacteria Thermus thermophilus and Thermus aquaticus possess approximately 21 and 24 proteins, respectively; the large subunits have about 35 and 37 proteins. This is about the same number as that of ribosomal proteins from Escherichia coli (11, 30). Most of the low molecular weight proteins from the two thermophiles are basic, as can be seen by their migration in the first dimension. The co-migration pattern (Fig. 13) shows a close relationship between the two organisms. Only small differences in the charge of corresponding proteins can be seen. This is not surprising since the 5S rRNA molecules from these two bacteria exhibit a homology of approximately 85% (74).

The protein extracts from the 30S and 50S subunits exhibit similarities to the corresponding extracts of Escherichia coli. These are differences in the charge of the r-proteins whereas their molecular weight range is almost the same among all three organisms (as deduced from the migration of the proteins in the second dimension).

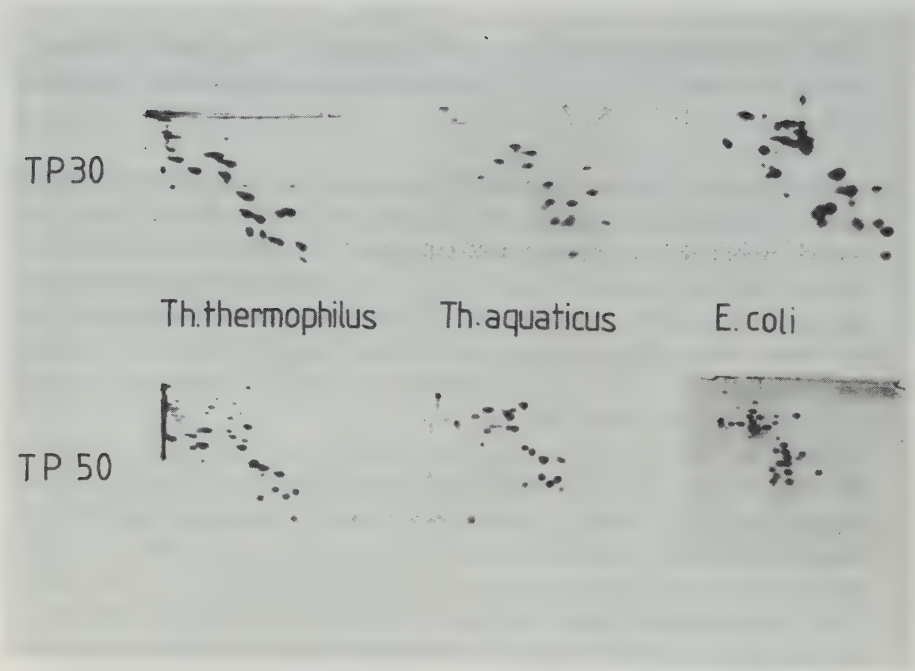


Fig. 51: Ribosomal proteins from Thermus thermophilus, Thermus aquaticus and Escherichia coli.

The small subunits of the Gram-positive thermophilic Bacillus caldolyticus and of the mesophile Clostridium pasteurianum have 24 and 25 proteins, respectively and the large subunit 30 and 34 proteins, respectively. This number is close to that given for the subunits of Bacillus stearothermophilus (24S and 34L proteins) and Escherichia coli (21S and 34L proteins). The 2-D-gel patterns of Bacillus caldolyticus and Clostridium pasteurianum exhibit differences mainly in the charge of the individual proteins, whereas differences in the molecular weight distributions are less evident. Proteins from the thermophilic organism are more basic (Fig. 14). The 2-D-gel pattern of Bacillus caldolyticus exhibit striking similarities to that of Bacillus stearothermophilus, as revealed by co-migration of proteins from the 50S and 30S subunits from the two organisms (Fig. 14). The similarity of the ribosomal protein patterns from the two thermophilic organisms is in good

agreement with the phenotypic and genotypic characterization of these organisms (97).

4.3. Functional studies on the DNA binding proteins

The isolated proteins were tested by different experiments for their ability to bind to nucleic acids. The Tsb-, Bcb- and Csb-protein bind tightly to DNA as was shown by a filter binding assay (3.5.1.) and gel filtration of protein/DNA complexes (3.5.2.2.). Furthermore, a strong affinity to various nucleic acids, such as 5S rRNA, 16S rRNA and synthetic polycytidylic acid, were observed. A strong affinity to ribosomes and subunits from the corresponding as well as from different organisms was seen. The same features have been reported for the DNA binding protein II from Bacillus stearothermophilus (48). The affinity of the proteins to ribosomes as well as to subunits could be a reason for the appearance of Asb-protein from Thermus aquaticus in the corresponding ribosomal protein extracts (Fig. 12). The protein was in a 3 - 4 times higher amount on the 50S subunit than on the 30S one, as determined by Coomassie blue staining. These results with Thermus aquaticus differ from those with E. coli (22) where a tight association of a similar DNA binding protein (NS-proteins) to the 30S subunit but a less strong affinity to the 50S subunit and to 70S ribosomes were observed.

The tight association of the isolated proteins to ribosomes and subunits is reasonable since topographical studies (82 - 86) have revealed regions on the surface of the ribosomes from Escherichia coli which are seemingly deficient in proteins and therefore contain exposed rRNA to which these proteins can bind. If there is a specific binding site for the proteins on ribosomes it should be possible to localize it by immune electron microscopy.

In order to investigate the Tsb binding to rRNA and DNA we have studied the physico-chemical properties of the nucleoprotein complexes by thermal denaturation experiments. Similar experiments on ribonuclease-DNA (88), gene-5-protein-DNA (89) and histone-DNA complexes (87, 90, 91), have revealed a destabilizing as well as a stabilizing effect after complex formation. These effects have been described as a result of different binding affinity towards native and denatured nucleic acid. Our experiments show that the Tsb-protein has a destabilizing as well as a stabilizing effect on

rRNA and DNA. The destabilizing occurs at the optimal growth temperature of Thermus thermophilus, whereas the stabilizing can be seen above this temperature. Therefore, this protein might possess an unfolding affect on nucleic acids which is probably important for their replication.

4.4. Structural studies on DNA binding proteins

The DNA binding proteins from various prokaryotic organisms have molecular weights of approximately 10.000. Intensive work has been done on the proteins (NS1 and NS2) from Escherichia coli which firstly were observed on native ribosomes (21, 22). Closely related proteins have been isolated from other eubacteria, such as Bacillus stearothermophilus (23, 48), Pseudomonas aeruginosa (26), Thermoplasma acidophilum (27) and Rhizobium meliloti (25). Therefore, it was of interst to look for structural similarities of the DNA binding proteins isolated in our laboratory to the corresponding proteins from other organisms. Our structural studies were performed on the level of primary, secondary and tertiary structure.

4.4.1. Primary structure

The N-terminal sequence analysis of the Tsb-protein reveals little homology to known sequences of the DNA binding protein II. The best similarity is seen with the Bsb-protein (11 out of 42 residues of the N-terminal sequence are identical). The Tsb-protein is characterized by a high degree of basic residues, such as lysine (approximately 15%). Since several tryptic and peptic peptides exhibit hydrophobic amino acids at the C-terminus, it was not possible to elucidate the whole sequence by manual Edman degradation. This problem might be overcome by the chemical cleavage of the protein at the methionine residue followed by isolation and solid-phase sequencing of the C-terminal peptide.

A comparison of the N-terminal sequences from 5 DNA binding proteins (Bsb, Bcb, Csb, NS1 and NS2) shows a rather strong similarity among them.

	1	2	3	4	5	6	7	8	9
Bsb	Met	Asn	Lys	Thr	Glu	Leu	Ile	Asn	Ala
Bcb	Met	Asn	Lys	Thr	Glu	Leu	Ile	Asn	Ala
Csb	Met	Asn	Lys	Ala	Glu	Leu	Ile	Thr	Ser
NS1	Met	Asn	Lys	Ser	Gln	Leu	Ile	Asp	Lys
NS2	Met	Asn	Lys	Thr	Gln	Leu	Ile	Asp	Val

Data for NS1, NS2 from (21, 73), data for Bsb from (23)

The analysis of the tryptic peptides of the Bsb- and Bcb-proteins shows differences in 4 peptides only (Section 3.6.2.3.4.). These results are supported by the co-migration of most Bsb- and Bcb-proteins on the 2-D-gel electrophoresis and by the PMR experiments. No differences between the two proteins could be seen by the two methods. The similarity of the primary structures reveals a close relationship of the two organisms.

The complete sequence analysis of the Csb-protein from Clostridium pasteurianum (92) clearly demonstrates a relationship of approximately 60% to the Bsb-protein and 46% to the NS-proteins. These results are also in good agreement with the homology among the 5S rRNA sequences from the three organisms (5). The 5S rRNA from Clostridium pasteurianum shows a stronger homology to the corresponding molecule from Bacillus stearothermophilus than to Escherichia coli.

4.4.2. Secondary structure

The stability of the secondary structure of the binding proteins was studied under the influence of temperature and urea treatment. The Tsb-protein shows a gradually diminution of the α-helical amount over a temperature range of 30° - 80°C and a cooperative helix-random-coil transition at 85°C, which is in good agreement with the maximal growth temperature of

Thermus thermophilus. The "melting" features of the Csb-protein are similar to Tsb, as it is reflected by a gradually diminution of the helix-coil transition over a temperature range from 30° to 60°C. A non-cooperative melting was observed around 53°C. The same features have been reported for the NS-proteins from Escherichia coli (48). In contrast, the Bcb-protein shows no alteration of the α-helical content up to 50°C, which is 17°C below the temperature optimum of growth. Melting occurs at 69°C. All three proteins show a well-ordered secondary structure which is stable up to the optimal temperature of growth. These results are confirmed by the urea denaturing experiments. The proteins renature completely when placed into the original environment.

4.4.3. Tertiary structure

The tertiary structure studies were performed by PMR spectroscopy and crystallization of the proteins. The PMR experiments on the salt-extracted proteins demonstrate a well-folded tertiary structure of the binding proteins, namely of Tsb and Bcb (Csb was not investigated since most of the protein was spent for sequence analysis and crystallization attempts). This is reflected by the resonances at 8 - 9.5 ppm, as well as by perturbed aromatic peaks in the low field region of the spectrum and ring current shifted peaks demonstrating a close neighbourhood of aromatic and hydrophobic residues. Such features are generally reported for folded proteins (35-37, 72, 93). Upon nucleotide binding, several alterations occur in the PMR spectra which are different in the two proteins. The Bcb-protein exhibits similar alterations on the arginyl resonances at 3.2 ppm as reported for the Bsb-protein. These residues are supposed to be involved in the binding to the sugar phosphate backbone of nucleic acids as postulated from a crystallographic analysis of Bsb-protein (75). No conformational changes of the protein tertiary structure were detected by PMR spectroscopy in the two protein-nucleotide complexes investigated.

The Tsb-PMR-spectrum reveals changes in the hydrophobic as well as in the amide proton resonances upon nucleotide binding. This is an indication for a partial conformational alteration. Hydrophobic amino acids should be engaged in either protein or nucleotide binding as is reflected by the diminution of the high-field resonances. These data are useful for further analysis of the action of Tsb-protein on nucleic acids.

Further investigations on the tertiary structure of Tsb and Bcb were carried out on crystallization and X-ray analysis of the protein crystals. The Tsb crystals have a resolution of about 3 Å, which is satisfactory for the determination of the three dimensional structure of the protein. The elucidation of the primary structure (which is in progress) will further facilitate these investigations.

The protein crystals of Bcb exhibit a similar shape as the corresponding crystals of Bcb. The X-ray analysis of the crystals is in progress and promises to be very successful (A. Koch, personal communication).

5. Summary

1. Ribosomal proteins from Thermus thermophilus, Thermus aquaticus, Bacillus caldolyticus and Clostridium pasteurianum were isolated and compared with the corresponding proteins from Escherichia coli and Bacillus stearothermophilus. The 2-D-gel electrophoresis patterns show a close relationship between Thermus thermophilus and Thermus aquaticus and a pronounced similarity of both to Escherichia coli. The ribosomal protein pattern of Bacillus caldolyticus is virtually identical to that of Bacillus stearothermophilus. Clostridium pasteurianum is more closely related to Bacillus stearothermophilus than to Escherichia coli.

2. A DNA binding protein with a molecular weight of approximately 10.000 was purified from four different organisms. The protein binds to DNA and to ribosomes as well as to subunits. No species-specific binding was observed. The N-terminal sequences of the DNA binding proteins from Thermus thermophilus (Tsb), Bacillus caldolyticus (Bcb), and Clostridium pasteurianum (Csb) were compared. Little homology of the N-terminal region (up to the 42nd residue) was observed between the Tsb-protein and the Bsb-protein. The Bcb- and Csb-proteins are homologous to the DNA binding protein II from Bacillus stearothermophilus and Escherichia coli.

3. The secondary and the tertiary structures of three proteins were investigated. The stability of the secondary structure of these proteins correlates fairly well with the growth temperature of the bacteria. Proton magnetic resonance spectroscopy studies of Tsb and Bcb indicate a compartly folded tertiary structure. Upon nucleotide binding, the Tsb-protein shows pronounced changes of the hydrophobic, and aromatic resonances. Nucleotide binding appears to induce small conformational changes. In contrast, the Bcb-protein, like the Bsb-protein, shows very little structural changes. In both cases, arginyl residues appear to be involved in nucleotide binding.

4. Crystals of the Bcb- and Tsb- have been obtained. The former ones appear to be virtually identical to those already described for Bsb. The properties of the Tsb crystals are described and some crystallogrpahic data are presented.

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